Costs and benefits of maternally derived immunity in a game bird system

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Submitted for the Degree of Doctor of Philosophy

The University of Edinburgh 2009

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Abstract

This thesis examines the costs and benefits of maternal allocation to both mother and offspring in gamebirds, specifically ring necked pheasants (*Phasianus colchicus*) and the Chinese painted quail (*Coturnix chinensis*). Maternal allocation of compounds from the mother to the young during early development can potentially have both positive and negative effects. For example the transfer of nutrients to the offspring can help to increase post-partum survival and subsequent life time reproductive success. In contrast, the transfer of stress hormones can potentially have long term negative effects on the offspring's development. The first part of this thesis investigates the effects of the transfer of immune components from mother to offspring, which are known to have positive short term effects (protection against pathogens in early development). There is therefore considerable interest in whether this response can be induced by maternal vaccination to confer protection to young birds in commercial situations. However, it is unclear how this affects life-history trade offs in the mother and the development of the offspring post-hatching. These effects are especially important in the gamebird industry where birds are commercially reared on a large scale before being released into the wild. Chapter 2 therefore examines the costs and benefits to the mother of producing a maternally transmitted immune response to the CoxAbic vaccine, such as the impact of vaccination on egg laying characteristics and body condition. Chapter 3 looks at these costs and benefits to the chicks by examining growth rates and survival after a challenge of live coccidia in offspring from vaccinated and unvaccinated mothers. Chapter 4 examines the immune response to vaccination in adult pheasants. Finally,

chapter 5 considers other factors that have been shown to affect the allocation decisions of females, specifically the effect of male characteristics on female reproductive effort.

Declaration

I declare that this thesis has been composed by me and is the result of my own work, except for the collaborations mentioned below. It does not exceed 70 000 words, and has not been submitted to any other university in application for a higher degree.

Chapters 2, 3 & 4: Ralph Marshall of the Veterinary Laboratories Agency assisted with the refinement of the oocyst extraction protocol, and microscopic identification of isolated *Eimeria*.

Chapter 4: Karen Fairlie-Clarke assisted with the running of the ELISAs and Andrea Graham and Karen Fairlie-Clarke assisted with the interpretation of the results.

Chapter 5: The experiments in this chapter were part of BSc (hons) projects planned by Emma Cunningham and carried out by Ellie Enright and Gillian Simpson. All analysis of the results were my work.

Matthew B. Ellis, 2009

Acknowledgments

There are many people who have proved invaluable in the production of this thesis.

Firstly I would like to thank my supervisor Emma Cunningham for her support, guidance, and unending patience. I wish her the best of luck with her growing lab group, and growing family.

I would also like to thank my other supervisors, Andrea Graham at the University of Edinburgh for her help with all matters immunological, and Rufus Sage and Chris Davis at the Game and Wildlife Conservation trust for their help with the pheasants. Mark Blaxter and Vernon French of the University of Edinburgh have also been a great help throughout.

For their technical expertise and guidance I also wish to thank Des Purdy for his help on the rearing field, and Karen Fairlie-Clarke for her help running and interpreting the ELISAs.

Despite acting as a slight distraction the ICAPB panto and EMPSEB conference were fantastic fun and great experiences. For the fun, excitement, unending committee/script meetings and the sheer hell of it I thank Max Burton, Ed Sykes, Nikki Brydges, Ali Duncan, Matt Robinson, Tom Reed, Helen Senn, Pedro Vale, Adin Ross-Gilespie, Tabitha Innocent, Eleanor Barnwell, Anjie Harris, Elie Dolgin, and Juan Carlos Ruiz-Guajardo.

On a personal level I want to thank all those who have helped or entertained me throughout my PhD, in no particular order, a selection of people from the University of Edinburgh including Matt Robinson, Tom Reed, Adin Ross-Gilespie, Pedro Vale, Tabitha Innocent, Eleanor Barnwell, Helen Senn, and Juan Carlos Ruiz-Guajardo, and from my new job the people that have helped me fit in the final push to finish my thesis, including Cathie Simister, Amanda Holroyd, John Harradine, Colin Shedden and Chris Brooks. Outside of academia and work Carl Rudd has helped to keep me motivated, James Kitson has provided me with some suitably exciting adventures, and some suitably regrettable home brew choices. My partner Rosie MacDiarmid has been a great proof reader, a fantastic and strict motivator when times were trying, a constant source of encouragement and pony-based relaxation, and of course the best of company.

Finally I wish to thank all those that have funded the last few years, including BBRSC, G&WCT, the Weir Fund for Field Studies, Abic and ASAB.

Chapter 1 - Introduction

1.1. Introduction

A fundamental aim in biology is to understand the factors responsible for variation in animal traits that influence the health and fitness of individual offspring. The phenotype of an individual is determined not only by the genetic contributions of the parents, but the environment the offspring are raised in and the environment the parents (especially the mother) experience during the development of the offspring. Conditions during early developmental and embryonic stages can have surprisingly strong effects on resulting offspring, leading to both positive and negative effects on survival and fecundity that can last into adulthood (Mousseau and Fox, 1998). These genotype-by-maternal phenotype interactions often explain a similar level of variation in a trait as the obvious underlying genetic differences (Cheverud and Moore, 1994). Measuring the impact of these effects is therefore essential to understanding biological processes across a wide range of disciplines, as well as determining the relative importance of different factors for offspring success.

1.1.1. Maternal effects

Maternal effects occur when the phenotype of a mother directly affects the phenotype of her offspring, independently of the offspring's genotype. The phenotypic maternal traits that cause these effects may have a genetic or environmental determined basis. For example, it is known that egg size in birds can potentially play an important role in determining offspring success (Pinowska *et al.*,

2004; Arnold *et al.*, 2006), but egg size has a genetic component and can also be affected by environmental variables as diverse as food availability (Wedell and Karlsson, 2003) and choice of partner (Cunningham and Russell, 2000).

Maternal effects are commonly considered to be by-products of maternal condition or the maternal environment (See Fox and Mousseau, 1998). For example, maternal host plant choice in gypsy moths (*Lymantria dispar*) can affect egg composition and subsequent growth and development of the offspring (Rossiter *et al.*, 1993; Rossiter, 1994) and maternal stress has been associated with reductions in hatchling mass and rate of plumage development in barn swallows (*Hirundo rustica*) (Saino *et al.*, 2005). However, stress hormones such as corticosterone, which are released as part of the stress reactions of many vertebrates, can potentially cause some of the negative effects discussed above, but are also essential to the development of the foetus (Liggins, 1994; Wada, 2008) and at various other times throughout development (Krug *et al*., 1983; Galton, 1990; Heath, 1997; Schwabl, 1999; Kern *et al.*, 2001; Seabury and Breuner, 2005). Furthermore, exposure of offspring to maternal stress hormones during development can also have other potentially beneficial effects post-partum, such as increasing begging behaviour (Kitaysky *et al.*, 2001; Kitaysky *et al.*, 2003; Wada and Breuner, 2008) and improving long term survival (Meylan and Clobert, 2005).

While many of these examples are assumed to arise through the passive transfer of factors related to maternal condition, it may also be possible for mothers to alter aspects of their current reproductive attempt in response to local cues such as

environmental conditions, disease or mate attractiveness. Females are expected to increase investment when the returns from reproduction will be high (Fisher, 1930). So, if, for example, male attractiveness is a signal of male "quality", either genetic quality in terms of any indirect genetic benefits he may confer or direct benefits such as resource holding ability for example, then females would be predicted to increase investment in reproductive attempts with attractive males. This is termed differential allocation and was first observed in zebra finches (Burley, 1986; Burley, 1988), where females were observed to increase brood size in response to perceived attractiveness in males.

More recently there has been an increase in the number of studies on differential allocation (Reviewed in more detail by Sheldon, 2000; Rutstein *et al.*, 2005a). For example, female barn swallows have been shown to increase reproductive investment for males with artificially elongated tails leading to a greater number of clutches per season and a greater number of fledglings (de Lope and Møller, 1993; Møller and de Lope, 1995; but see Witte, 1995), female zebra finches have been found to alter hormonal levels in their eggs in response to perceived attractiveness of males (Gil *et al.*, 1999), female mallards have been found to alter investment in eggs associated with preferences for particular males with subsequent consequences for chick condition (Cunningham and Russell, 2000) and female blue tits have been shown to decrease feeding rate in response to males with artificially reduced crown chroma (Limbourg *et al.*, 2004) showing a possible negative response to perceived lower male attractiveness. However, there are possible costs to the mother associated with responding in this way. For example, increased reproductive effort, such as an

increase in clutch size, can potentially lead to reduced overwinter survival, such as that observed in blue tit mothers (de Heij *et al.*, 2006), and to potentially negative effects on the immune system, such as increased susceptibility to ectoparasites and reduction in the humoral immune response as observed in the collared fly catcher (*Ficedula albicollis*) (Nordling *et al.*, 1998). Nevertheless, apparently costly mechanisms can evolve if the costs to the mother are offset by the increased fitness of offspring. One candidate mechanism is the case of a specific maternal effect, maternally derived immunity.

1.1.2. Maternally derived immunity

The transfer of immune components from mother to offspring is a specific maternal effect that can significantly affect the responses of offspring to pathogens they encounter in early life and ultimately impact on their short term survival (Gustafsson *et al.*, 1994; Pihlaja *et al.*, 2006). If during gestation or egg production, a mother experiences an immune challenge by being exposed to some pathogen, then she will generally respond by mounting an immune response as a defence. These immune components can, in turn, be transferred to the offspring (Brambell, 1970; Baintner, 2007), for example, across the placenta (Bruce-Chwatt, 1954; Simister, 2003), via milk (Adler and Foner, 1965; Sadeharju *et al.*, 2007) or through different egg components (Rose, 1972; Pastoret *et al.*, 1998; Boulinier and Staszewski, 2008). There is also potential for the transfer of other potentially beneficial compounds such as antioxidants which can have positive effects on the innate immune system (Blount, 2004).

Maternally derived immunity is potentially beneficial to offspring as the immune system of neonates is not fully developed and so maternal immunity can provide them with temporary immunity against pathogens (Rose, 1972; Smith *et al.*, 1994b; Hassan and Curtiss, 1996; Al-Natour *et al.*, 2004) whilst their own immune system develops (Muggli *et al.*, 1984; Apanius, 1998). This can reduce the cost to the offspring associated with mounting an immune response and allow investment in other life history traits. For example, a recent study of *Coturnix japonica* (Grindstaff, 2008) found offspring challenged with the same immune challenge as their mothers showed a higher growth rate compared to challenged offspring from unchallenged mothers. Similarly, great tit mothers exposed to the hen flea (*Ceratophyllus gallinae*) produce an antibody response which can be transferred to the offspring (Buechler *et al.*, 2002), and when offspring from mothers that were challenged during egg laying were themselves challenged they showed higher growth rates and adult weight, were more likely to survive to fledging and were more likely to produce offspring themselves than challenged chicks from unchallenged mothers (Heeb *et al.*, 1998).

There is also some evidence that the presence of these maternal immune components in the offspring can be beneficial even in the absence of any infection, for example, in the same great tit system but with cross fostering between challenged and unchallenged mothers it was found that chicks from challenged mothers raised in low parasite environments were heavier and in better condition than chicks in the same environment from unchallenged mothers (Gallizzi *et al.*, 2008). The authors propose several mechanisms for this including enhanced protection against transient ectoparasites such as blowflies, or transfer of “immune-activating substances” like

antioxidants that can have positive effects on offspring growth and survival (Blount, 2004). However, it is also possible that challenged mothers simply increase investment in factors unrelated to immunity in their offspring in order to "prepare" them for the expected environment.

Local conditions (for example vector prevalence) can have a strong effect on the amount and timing of antibodies transferred to young by mothers (Gasparini *et al.*, 2001; Staszewski *et al.*, 2007). Studies on the black-legged kittiwake (*Rissa tridactyla*) for example, have shown inter-annual effects where the mother's immune response from one year is transferred to offspring in subsequent years. Furthermore, the levels of circulating antibodies in the mother correlated with the levels in the egg and even the adult offspring (Gasparini *et al.*, 2002) suggesting a possible mechanism for maternally derived immunity where the mother transfers a proportion of her current immune levels to her offspring. However, this does not remove the possibility that the difference in observed immune response is an inherited genetic difference, rather than evidence of passive transfer of immune components to the eggs.

While it may be beneficial to protect offspring through a mechanism such as maternally derived immunity it is possible that there are associated costs. For example, there may be costs of producing extra immune components to the mother (Kowalczyk *et al.*, 1985) and possible long term costs to the offspring due to, for example, interference from the mothers immune response with their own. A blocking effect of maternal immune components has been inferred in studies examining the

effects of maternal immunity in poultry. Maternal vaccination against infectious bursal disease virus (IBDV) has been used in chickens to produce a maternally derived immune response in offspring (Guittet *et al.*, 1982; Negash *et al.*, 2004). This has possible beneficial effects in that it can potentially stimulate numerous immune responses in the developing embryo which may result in production of anti-IBDV antibodies and possibly other immune components. However, maternal vaccination with an IBDV immune complex or IBDV-2512 vaccine also been shown to restrict the neonates ability to react to *in ovo* vaccination with the same vaccine (Corley *et al.*, 2002), though the mechanisms of this are unclear.

A possible mechanism has been exposed in a rodent malaria system using a novel subunit vaccine that produces a maternally transmissible response. The maternal components produced in response to the vaccine, and passed to the offspring, appear to block the offspring's response preventing the pup from mounting its own immune response to the vaccine for up to two weeks, with the strength of its response increasing as the maternal antibodies wane (Good *et al.*, 2004). However, this blocking response is only limited to the antibody producing part of the immune response (Sedegah *et al.*, 2003). A possible explanation for this may be a competitive exclusion mechanism, whereby the maternal antibodies bind all available sites on the vaccine components, thus preventing binding of offspring antibodies to these sites and blocking the offspring's ability to react to the vaccine antigens.

Assessing the costs and benefits of maternally derived immunity is complicated, but has recently been comprehensively reviewed (Hasselquist and Nilsson, 2009). On the

one hand offspring are protected from disease by maternally derived antibodies so the offspring's ability to mount an immune response of their own is reduced, and they presumably have more energy to invest in growth and development (Buechler *et al.*, 2002; Martin *et al.*, 2003). This potentially has important long term effects on offspring fitness (Keeler and van Noordwijk, 1993; Gebhardt-Henrich and Richner, 1998). On the other hand, there may be compensatory costs associated with any increased growth rate (Gebhardt-Henrich and Richner, 1998; Blanckenhorn, 2000; Fischer *et al.*, 2004) due to altered investment as a result of being unable to mount an immune response. For example, increasing the metabolic demands of nestling birds, and thereby increasing its chances of starvation, or leading to damage at the cellular level (Mangel and Stamps, 2001). Whilst the potential costs of maternally derived immunity to the mother may be important it is assumed that overall the effect is positive and that maternally derived immunity ultimately has a beneficial effect for both parent and offspring fitness through effects on the offspring's fitness, and consequently on parental inclusive fitness.

It is likely that there are costs associated with the transfer of maternally derived immunity, as described above, and so it is also likely that it is subject to similar pressures as other maternal effects. Namely, that a female with limited resources is expected to increase investment in the current reproductive attempt when the expected returns are high (Fisher, 1930). Differential investment of immune components is another possible way of increasing investment in the current reproductive attempt, if the cost to the mother of transferring immune components is offset by the benefits to her offspring, such as increased survival. There are a number

of studies that have examined this, for example female barn swallows have been found to increase transfer of antibodies to their eggs when mated with males with artificially elongated tails (Saino *et al.*, 2002b). However, this is in contradiction to a similar study in collared fly catchers that found no effect of unmanipulated male characters on female transfer of immunity the eggs (Hargitai *et al.*, 2006), though this study was correlational, not experimental. If these effects occur outside of experimental systems then they are potentially another important way for females to alter reproductive investment in response to male characteristics and may be important in controlling offspring success.

As previously alluded to, a strong immune response at the right time, such as that induced by vaccination during gestation, can lead to the transfer of maternal immunity, providing a potentially useful mechanism to protect young animals being reared commercially (Pravieux *et al.*, 2007). The ability to vaccinate one individual and have them pass on their immunity to their offspring has obvious applications for animal husbandry, and in particular the game bird and poultry industries where the number of offspring produced per female are high.

1.1.3. Maternally derived immunity as a vaccination strategy

Maternal vaccination, as well as providing protection to the mother, can also induce protection in her offspring through the mechanism of maternally derived immunity. Therefore, rather than relying on an infection induced immune response in the mother the maternal immune response can be induced through vaccination. There are a number of vaccines specifically designed to provide neonatal protection via this

mechanism, for example Bovilis BVD (Intervet UK Ltd) based on cytopathogenic bovine viral diarrhoea virus (Donovan *et al.*, 2007; Platt *et al.*, 2008), Eurican Herpes 205 (Merial Animal Health Ltd) which provides neonatal protection against herpes virus in dogs (Poulet et al., 2001) and CoxAbic (Abic Biological Laboratories Teva Ltd, Israel) which reduces *Eimeria* oocyst output in commercial chickens and their offspring (Wallach, 2001; Ziomko *et al.*, 2005).

There has been considerable interest in the CoxAbic vaccine due to the importance of *Eimeria* in the poultry industry. Coccidiosis, caused by the protozoan *Eimeria*, is economically a very important disease which was estimated to cost around £38.5m to the worldwide poultry industry in 1995 (Williams, 1999) with more recent estimates at $1.2-$2 billion annually (V. Shriker, Abic, pers. comm.). Traditionally coccidiosis has been controlled through the use of coccidiostat feed additives. However, new legislation governing the use of feed additives in the livestock industries means there is an increased focus on developing cheap and effective vaccines. CoxAbic is one such vaccine. CoxAbic is a subunit vaccine containing specific proteins from the wall forming bodies of *Eimeria maxima*, which are important in forming the environmentally resistant oocyst wall. Despite the species specific nature of *Eimeria* CoxAbic has been shown to be effective at reducing oocyst output in mothers and offspring challenged with a number of different *Eimeria* species in chickens (Wallach, 2001; Ziomko *et al.*, 2005) and unpublished trials in pigeons (M. Wallch, pers. comm.). It is likely that this broad response is due to the conserved nature of the wall forming bodies on which it is based. This highlights the potential to use this

vaccine in other avian breeding systems where coccidiosis is problematic. One such area is the breeding of game birds.

1.1.4. Maternally derived immunity as a vaccination strategy in game birds

Game birds are defined under the Game Act (HMSO, 1831) as "*any pheasant, partridge, grouse (or moor game), black (or heath) game or ptarmigan*". Traditionally wild populations of lowland game birds are supplemented by the yearly release of field reared stocks to maintain a population for shooting. These birds form an important part of the contribution of countryside sports to the rural economy which is estimated to generate £2 billion, provide 70 000 jobs and actively manage and conserve 15 million hectares of land in the UK (PACEC, 2006).

Game birds are generally long lived in the wild and so it is important to understand which factors can potentially influence their long term fitness. Pathogens are one such important factor (Dobson and Hudson, 1986; Hudson and Greenman, 1998; Millan *et al.*, 2004; Hudson *et al.*, 2006). Infection with the nematode *Heterakis gallinarum*, for example is considered to be important in regulating competition between populations of pheasants and grey partridge (*Perdix perdix*) and subsequently population levels of both species (Tompkins *et al.*, 2000; Tompkins *et al.*, 2001). However, later work disputes these findings and suggests that population level effects may only occur under limited conditions (Sage *et al.*, 2002).

One of the most important gamebirds to the rural economy is the ring-necked pheasant (*Phasianus colchicus*) which is often reared artificially in large numbers around the UK and then released to supplement wild populations, with an estimated 20 – 30 million pheasants being released each year (Davey and Aebischer, 2008). Pheasants were introduced into Europe from their native habitat of the Himalayan foothills by the Romans, and then from naturalised European populations into England in the 11th century by the Normans. It has become an important game bird, with a steady reintroduction both from British and French game farms every year to maintain shooting stocks. Pheasants are highly sexually dimorphic with the males being brightly coloured with conspicuous red wattles. In the wild males establish breeding territories in early spring and fight to defend harems of up to 16 females, who will lay up to 12 eggs each. Pheasants require woodland and scrub fringe with long grass for roosting and nest making, and field fringes offer the best habitat for the diverse array of plants that support the proteinaceous insects needed to raise the young (Wagner *et al.*, 1965).

Pheasant rearing to supplement these populations begins every year in January, often with the catching up of wild birds, though some flocks are overwintered in captivity. These birds are placed in laying pens and their eggs collected and incubated throughout the season. At the end of the laying season the laying hens are often released back into the wild. At all stages precautions are taken to reduce the stress to the birds. This includes keeping stocking density at reasonable levels which helps to reduce the effect of the birds on the environment and reduces the prevalence of cannibalism and pathogens within the population. Despite these measures the birds

are still raised in higher than natural densities which inevitably increases the prevalence of pathogens. Common pathogens of reared pheasants include *Mycoplasma* sp., the nematode *Syngamus trachea* (otherwise known as gapeworm) and the protozoans *Spironucleus meleagridis* and *Eimeria* (also called Coccidia).

Due to the costs associated with disease in intensively reared birds, both in economic and welfare terms, it is necessary to implement control measures against these pathogens, and as such maternal vaccination has the potential to be very useful in the gamebird system. In the case of coccidiosis, long term prophylaxis with coccidiostat feed additives can control infection, and treatment with drugs such as Baycox (Toltrazuril, Bayer HealthCare) can effectively treat an outbreak; however it is possible that the flock will need several treatments throughout the time they are being reared if further outbreaks occur. Furthermore there are practical and legal difficulties in administering such drugs once the birds are released. Maternal vaccination could therefore be a more economical alternative as vaccination of the mothers can provide protection for her numerous offspring and the vaccination regime is likely to only be needed once in the bird's lifetime.

Unlike the broiler population of the poultry industry, birds reared in the game industry often live for several years in the wild after being released rather than being slaughtered at a month or two of age. Therefore the long term costs and benefits of maternal vaccination which are relatively unimportant in the poultry broiler industry, such as long term immune responsiveness, become very important in the game industry and can potentially have a large impact on the survival prospects of released

birds and their offspring. In order to understand and assess these costs and benefits it is necessary to have a better understanding of the mechanisms involved at every level. For example, the costs and benefits of producing the maternal response to the mothers, the costs and benefits of receiving the maternal response in the offspring, and how this response interacts with the offspring's own developing immune system and trades off with other life history traits. The aim of this project is to assess the importance of these potential positive and negative effects and to examine the viability of utilising specific maternally derived immunity vaccines developed for the poultry industry to increase offspring survival levels in game farm systems.

1.2. Aims

This study aims to examine the positive and negative effects in mothers and offspring of stimulating maternally derived immunity via vaccination in a game bird system. This is achieved by using one main experimental framework where a single experiment was repeated across two years using phosphate buffered saline (PBS) as a placebo and the CoxAbic vaccine as the treatment groups in both years. However, each repeat also examined additional factors, with the first repeat examining the effect of different components of the vaccine and the second repeat exploring the effect of rearing substrate. Therefore the experiments reported in chapters 2, 3, and 4 use the same birds, and some parts of the experimental methods overlap. However, each chapter examines an independent and specific aspect of the costs and benefits of maternal vaccination. Chapter 2 examines the costs and benefits to the mother of producing a maternally transmitted immune response to the CoxAbic vaccine, such as the impact of vaccination on egg laying characteristics and body condition.

Chapter 3 looks at the benefits to the chicks by examining growth rates and survival after a challenge of live coccidia in offspring from vaccinated and unvaccinated mothers. Chapter 4 examines the immune response to the CoxAbic vaccine in adult pheasants. Finally, Chapter 5 looks at the possible effects of male traits on differential allocation in the pheasant and quail and how these may affect egg laying and important life history characteristics.

Chapter 2 - Costs and benefits of the CoxAbic vaccine to laying hens

2.1 Introduction

Ever since Edward Jenner and Louis Pasteur began experimenting with vaccination in the late 18th and early 19th century there has been considerable debate over its costs and benefits. In livestock systems, vaccination and the associated protection from disease should lead to greater productivity (for example increased yield, or increased food conversion) compared to non-vaccinated systems, better welfare for the livestock, as well as reduced costs to the farmer (one vaccination course instead of a lifetime of prophylactic and therapeutic drugs). However, there are also possible trade-offs associated with vaccination, in terms of the side effects of the drug, the cost of mounting an immune response to the vaccine and the beneficial protection it may produce. More long term and potentially devastating costs include the loss of herd immunity and the possible re-emergence of the disease due to antigenic variation.

Maternal vaccination induces the same sort of immune response in the mothers as normal vaccination and is therefore likely to have similar costs and benefits, both in terms of economics and its impact on individual fitness traits. However, it differs in that the extent of protection conferred may be much greater in terms of the number of animals in a population that are protected. In order to assess the costs and benefits of maternal vaccination it is necessary to understand the costs and benefits to the mothers as well as the potential benefits to offspring.

The aim of this chapter is to examine the costs and benefits of vaccination on economically important female traits including egg characteristics (for example weight and volume) and female weight and condition, as well as the ability of the vaccine to provide protection in the face of a live challenge by a pathogen.

2.1.1. The costs and benefits of immunity

It has long been assumed that raising an immune response must have associated costs, and that these costs must be paid by trading off against other life history traits such as reproduction, survival or other costly activities (Råberg *et al.*, 2002; Saino *et al.*, 2003a; Boughton *et al.*, 2007; French *et al.*, 2007a; French and Moore, 2008; Zala *et al.*, 2008). This has been shown in a number of species including birds (Hanssen *et al.*, 2004; Boughton *et al.*, 2007), mice (Demas *et al.*, 1997) and invertebrates (Sadd and Siva-Jothy, 2006; Little and Killick, 2007). These potential costs are split into at least two main parts (But see Colditz, 2008). Firstly there is an energetic cost to producing the immune components (Demas *et al.*, 1997; Martin *et al.*, 2003; Amat *et al.*, 2007), and secondly there is a physiological cost due to the damage caused by the activated immune system (Sadd and Siva-Jothy, 2006), for example caused by non-specific immune responses such as fever and cytotoxins. The obvious assumption in the case of vaccination is that the cost of the immune response is less than that of the damage to host by an unchallenged pathogen. However, costs associated with vaccination are obviously important to consider, especially when assessing any potential net benefit to maternally derived immunity.

Some of these costs in birds have been inferred from changes in basal metabolic rate (BMR) in response to challenge. These are important as increases in BMR can potentially lead to greater energetic demands, and greater cellular damage. Martin *et al.* (2003) showed an increase of 28.8% in mass corrected resting metabolic rate of house sparrows (*Passer domesticus*) on injection with phytohaemaglutinin (PHA), a plant compound commonly used to stimulate a cell mediated immune response in vertebrates, suggesting that mounting an immune response to this compound was energetically expensive. Similar effects were also seen in larger studies such as that by Ots *et al*. (2001), who used a larger population (n = 42, but only 21 used for BMR study) of blue tits (*Parus caeruleus*) and found an 8.6% increase in basal metabolic rate (BMR) in recaptured challenged birds (challenged with sheep red blood cells) producing an antibody response compared to sham injected and recaptured bids. Finally a study by Bonneaud et al. (2003) found that inoculation of house sparrows with *E. coli* lipopolysaccharide (LPS) led to reduced activity and body mass, as well as reduced feeding effort of nestlings (leading to lower reproductive success), suggesting that energy was being diverted away from body maintenance towards mounting an immune response.

However, numerous studies have also seen no effect of mounting an immune response on life history trade-offs. Another study of house sparrows (Lee *et al.*, 2005) found no effect of PHA on BMR. The authors suggest this could be due to the experiments taking place at different times in the year, and differences in experimental design leading to a housing effect. Furthermore, although Svensson et al. (1998) found a non-significant increase in BMR of 8-13% in blue tits treated with

the diphtheria-tetanus vaccine, the authors suggest this may be due to small sample sizes. Interestingly, the same paper showed that birds housed in cold conditions produced lower antibody responses than birds in normal conditions which could suggest the possibility of a condition-immunity trade-off where investment in immunity is reduced to maintain necessary levels of body condition or a blocking effect of an acute stress reaction on the immune system.

These studies are important as they help to clarify the context for immunity based trade-offs. For example, the disparity between the Lee et al. (2005), Martin et al. (2003) and Bonneaud et al. (2003) studies serve to emphasise that these trade-offs may only occur when a resource is limited (such as food in the winter), or when the individual is under extra stress (for example during reproduction, or winter).

As well as possible effects on BMR the costs of immunity can also manifest as a trade off with another costly trait, reproduction. This has also been seen in a number of species, including birds (Ilmonen *et al.*, 2000; Buchanan *et al.*, 2003; Saino *et al.*, 2003a; Boughton *et al.*, 2007) and lizards (French *et al.*, 2007b; French and Moore, 2008). French's work on lizards (French *et al.*, 2007b; French and Moore, 2008) is important as it stresses the importance of resource limitation on trade-offs, as no trade off was observed between wound healing and reproduction under conditions of unlimited food (French *et al.*, 2007b). However, once food was restricted the trade off between reproduction and immunity became apparent with wounded, food restricted females producing significantly smaller follicles (egg precursors) than non-wounded, food restricted females. Furthermore females housed under *ad libitum*

laboratory conditions did not show a trade-off between wound healing and reproduction whereas females in the wild did, and vice versa in males (French and Moore, 2008). The authors speculate that there are two potential mechanisms behind this sex difference. The laboratory based individuals were fed *ad libitum* which potentially allowed the females to invest optimally in both traits, and laboratory based males were housed individually and so did not have to invest energy in costly behavioural and dominance related display that naturally occur in the wild but which were removed in the laboratory by housing individually. This particular example again serves to demonstrate the potentially important role of resource limitation on life-history trade-offs.

Studies looking at the trade-offs between immunity and other life history traits, such as reproduction often chose to use novel antigens that the individual will not have experienced before, for example sheep red blood cells, and human diphtheria-tetanus vaccine which are often used in birds such as blue tits (Råberg and Stjernman, 2003) and barn swallows (Saino *et al.*, 2000). By using novel antigens there is no individual variation in previous exposure levels as no members of the population will have encountered it. Therefore any difference in responses is due to factors such as differences in condition or immunocompetence rather than differences in individual exposure levels. This is more informative when trying to assess the level of costs that are attributable to the host immune response, rather than pathologies associated with the parasite.

However, in the normal situation where vaccines are used there is a range of previous parasite exposure and immunity within the population and a naïve individuals ability to mount a response to a given vaccine may not be a good predictor of the response achieved in a population with a spectrum of previous exposure and immunity levels. It is therefore important to understand the costs and benefits of vaccination in the face of prior pathogen history in order to assess the economical and ethical implications of vaccine use for disease control in natural and semi-natural systems.

In this study we will use the CoxAbic vaccine as an immune challenge for the mother. Coccidiosis is very common on rearing fields and so it is likely that most individuals will have built up some degree of protection against it. Also coccidiosis has coevolved as a parasite of galliformes such as pheasants, and so the pheasant immune system may have evolved ways of dealing with coccidiosis infections over their evolutionary history. Therefore challenge with a coccidian-derived vaccine is biologically relevant to the pheasant's natural history and so is likely to produce realistic responses to infection and will provide relevant data for interested parties who work to assess its effectiveness and economic viability as a protection method in avian breeding systems.

2.1.2. Coccidiosis: Biology and methods of control

Coccidiosis is caused by the apicomplexan parasite *Eimeria* and is common on intensive game farms. However, it rarely causes a significant amount of mortality unless the challenge is large enough, the birds are stressed or they become infected with another parasite. For example, it is now common practise to "bit" or "spec"

young pheasant chicks to prevent them from pecking at other birds in the pens. This procedure involves a lot of handling and due to handling stress can lead to outbreaks of coccidiosis in the flock.

The term Coccidiosis refers to infection with members of the Sub-Class Coccidiasina which are all pathogens of the intestinal tract and includes several important disease causing organisms including *Toxoplasma, Isospora, Sarcocystis* and *Cryptosporidium*. Poultry coccidiosis specifically refers to the genus *Eimeria*. This genus is both very host specific and very site specific within the gastrointestinal system and so it is possible for individuals to be infected with several species of *Eimeria* at different locations along the intestinal tract. In the UK there are three major species of *Eimeria* that infect pheasants. The most common of these is *E. colchici* (Norton, 1967; Jones and Wood, 1968), this species tends to infect the caecae and lower intestines. The remaining two species are *E. duodenalis* which tends to remain high in the intestinal tract, and *E. phasiani* which is predominantly found in the lower intestines. Coccidiosis is a challenging pathogen to manage because of its tendency to produce low level, almost asymptomatic infections that seed the environment with large numbers of oocysts. The infection is then spread throughout the flock causing little pathology until the birds become stressed or infected with another pathogen, leading to acute coccidiosis.

The *Eimeria* life cycle (Figure 2.1) involves several rounds of asexual reproduction within the host (merogony, or schizogony) and an environmentally hardened cystic stage called oocysts. Oocysts are excreted in the faeces and develop in the faecal

mass into infective sporulated oocysts. When ingested the oocysts rupture releasing sporocysts which in turn rupture to release sporozoites that invade the gut epithelial cells (invasion). Live attenuated vaccines act from this stage onwards. Once inside a host cell the sporozoites develop into meronts/ schizonts that undergo a round of asexual reproduction (merogony/ schizogony) that destroys the host cell and releases merozoites into the lumen, followed by more rounds of merogony. After the last stage of merogony most merozoites differentiate into macrogametes (female) with some forming microgametes (male) that undergo several fissions to release highly motile microgametes. These exit the host cell and fertilise the intracellular macrogametes forming a zygote (gamogony). The zygote produces wall forming bodies in the cytoplasm which in turn form the tough oocyst wall. Once the oocyst is fully formed the host cell is lysed. The CoxAbic vaccine acts as the oocyst is forming, making the CoxAbic vaccine a transmission blocking vaccine in the adults and offspring (Wallach, 1997).

Recent work has shown that small doses of live coccidia are able provide protection against *Eimeria colchici* in pheasants (Liou *et al.*, 2001). In this study two week old chicks were challenged with either distilled water or 100, 250, 500, 1000 or 2000 sporulated oocysts, then 18 days after inoculation were challenged with a large dose of 120 000 oocysts. During the following twelve day observation period 82% of un-inoculated chicks died whereas mortality in the inoculated groups decreased with increasing inoculation dose (7% mortality when inoculated with 100 oocysts, 4% mortality when inoculated with 250 oocysts, and no mortality in any other group).

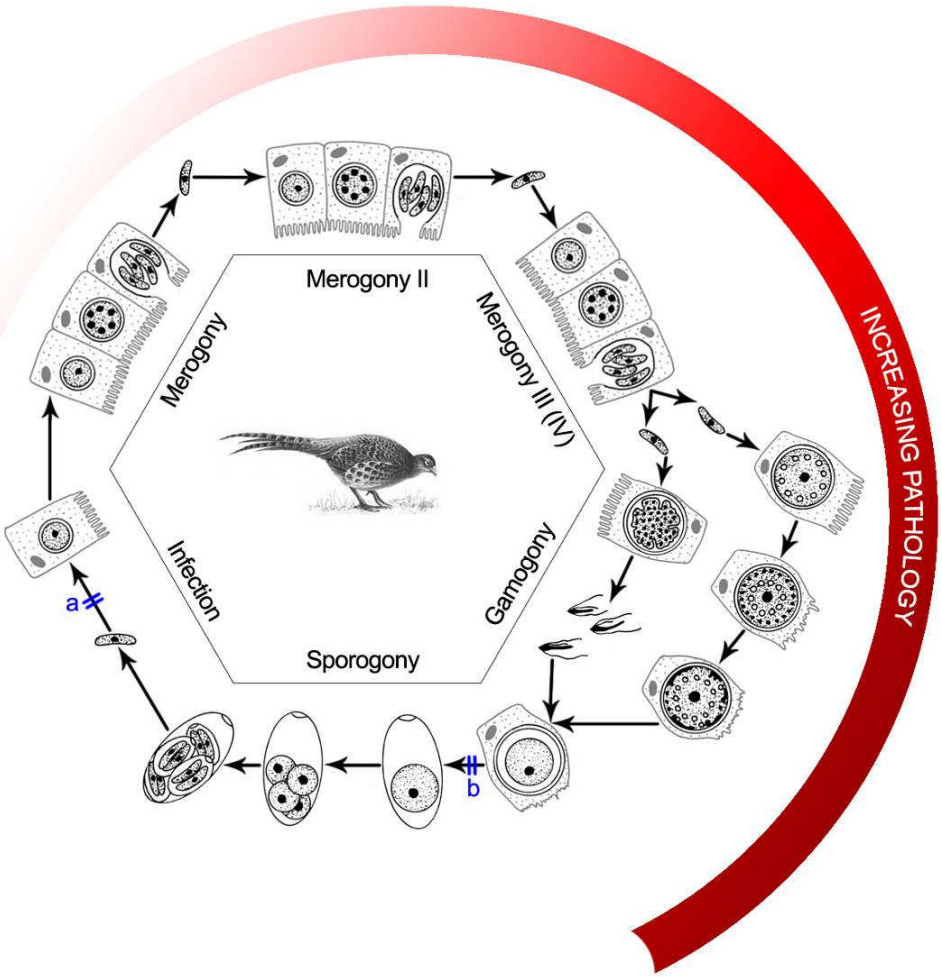

Figure 2.1: Overview of the life cycle of Eimeria. Blue text indicates intervention points for specific vaccine types. a: Live attenuated vaccines. b: CoxAbic subunit vaccine

The use of live coccidia is obviously unlikely to be widely popular in the poultry industry. However, it is also possible to use live-attenuated organisms as a basis for vaccination. This usually involves irradiating or heat treating the pathogen in such a way as to stop it from causing pathology in the host. This method of vaccination is beneficial as it stimulates a "real" immune response to the parasite at the locations it is likely to naturally infect. By attenuating the organism you are able to induce a normal immune response, without causing significant pathologies. For example, it is possible to induce partial immunity against *Eimeria* using live attenuated oocysts (Crouch *et al.*, 2003), therefore blocking invasion of the hosts epithelial cells. This study found that vaccinated and subsequently challenged birds did not differ significantly in growth rate from unvaccinated, unchallenged birds, but had a significantly higher growth rate than unvaccinated, challenged birds, showing no measurable negative effect on growth of the vaccine under these conditions., but a significant protective effect However, in the poultry industry this type of vaccine has potential negative effects as it may divert energy away from laying or growth (Kopko, 1998) reducing economically important factors such as feed conversion rate (Danforth, 1998; Crouch *et al.*, 2003). Another, though rare, problem is that the parasite may revert to its virulent form in the host (Cizman *et al.*, 1989; Christensen *et al.*, 1992; Minor, 1993; Nielsen *et al.*, 2001) and lead to the pathogen infiltrating and infecting the system. In intensive poultry farms this could quickly lead to large scale epidemics.

In terms of developing a vaccine, oocysts are only fleetingly intracellular and so it is difficult for the immune system to mount a quick and effective response, therefore

targeting of other intracellular stages of *Eimeria* may be more likely to succeed. However, targeting of intracellular stages is complicated by the fast cellular turnover during merogony, the asexual proliferative stage of the parasite. The gametocyte is the sexual stage of *Eimeria* and is a relatively slow stage in the parasite's life cycle, needing time to differentiate and encyst, and so is potentially the most accessible life stage. Whilst targeting the gametocytes cannot stop the infection and its associated pathologies (See Figure 2.1), it can block its transmission. One oocyst, containing eight sporozoites can produce over 2.5 million oocysts, therefore targeting the gametocyte stage and reducing the animals oocyst output will help reduce the levels of environmental infection (Levine, 1961), thereby blocking or significantly reducing any reinfection.

Earlier work on *Eimeria acervulina* merozoite and sporozoite apical complex antigens in poultry (Sasai *et al.*, 1996; Constantinoiu *et al.*, 2003; 2004) was promising as blocking invasion and merogony would also help prevent the vast majority of associated pathologies, as well as blocking transmission. Furthermore these antigens are conserved across a number of species of *Eimeria* (Constantinoiu *et al.*, 2003) and so would help in the development of a broad spectrum coccidial vaccine. However, more recent work has focused on the development of gametocyte based vaccines. For example, a recent study in chickens found that a crude gametocyte based vaccine produced a better protective response than a live attenuated vaccine (LivaCox. Biopharm, Czech Republic) (Anwar *et al.*, 2008a; Anwar *et al.*, 2008b). This was in a field experiment with no experimental, but presumably some degree of environmental challenge, as well as a laboratory

experiment with live challenge with mixed *Eimeria* species. This study found that treatment with the gametocyte extract compared to LivaCox or PBS led to greater circulating antibodies, a lower oocyst output following live challenge, and higher growth rates, final weight and lower mortality in field and laboratory experiments showing that the gametocyte vaccine was effective in reducing at least some of the costs associated with coccidiosis.

Other studies with *Eimeria* (Smith *et al.*, 1994b; 1995a; 1995b; Wallach, 2001) have led to the development of a subunit vaccine from *Eimeria maxima* gametocytes (CoxAbic) that can result in the transfer of maternally derived antibodies. As this is based on gametocyte antigens this vaccine acts as a transmission blocking vaccine, in that it does not stop infection of birds (if they ingest sporulated oocysts they will excyst and infect the epithelium), but it does prevent the parasite from seeding the environment with oocysts and provides protection against future challenge. Furthermore, this vaccine is based on two specific antigens found in the wall forming bodies of the gametocytes that are essential for it to encyst and become resistant. By injecting these subunits, together with an adjuvant, an antibody response is stimulated that initial tests have shown can provide a lasting response to those specific antigens that can also potentially be passed on to their offspring to confer protection.

2.1.3. The CoxAbic vaccine

Most subunit vaccines, such as CoxAbic, are made of the antigen of interest and an adjuvant. The adjuvant is used to stimulate a strong innate immune response which

ensures activation of the humoral response and prompts antibody production in response to the antigen of interest. The antigen of interest is usually an antigen that has been found to produce a protective immune response against the pathogen. By injecting the subunits, rather than the whole organism, an immune response is stimulated without the associated disease pathologies. This then leads to formation of memory B-cells which will more rapidly induce antibodies during any subsequent invasion by a pathogen with the antigen of interest.

The CoxAbic vaccine is composed of Freund's Incomplete Adjuvant (FIA) and uses the wall forming bodies from *Eimeria maxima* gametocytes as the antigen. Freund's adjuvant is available in complete (FCA) and incomplete (FIA) form. Both forms contain mannide monooleate and paraffin oil. These cause activation of the innate immune system without containing any specific antigens, and are important in amplifying the response to the specific antigens included in the vaccine and prolonging the antigenic stimulation by slowly releasing the antigens into the surrounding tissue. Freund's complete adjuvant also contains desiccated *Mycobacterium butyricum* which helps to activate the immune system further. Freund's complete adjuvant has the potential for serious side effects including localised necrosis and extreme pain, and so Freund's incomplete adjuvant is more normally used in modern vaccines. Early testing of the CoxAbic vaccine used Freund's complete adjuvant, but the commercially available form now uses Freund's incomplete adjuvant.

Initial data on the CoxAbic vaccine in chickens is based on trials using Affinity Purified Gametocyte Antigens (APGA; These are the subunits that were eventually selected to be used in the vaccine). The original data for these trials is presented as originally shown in Table 2.1, but see Figure 2.2 for a graphical representation. In terms of the effect on chick oocyst excretion it would appear that the effect of the APGA is no greater than that of FCA in PBS, though both showed reductions relative to the control treatment.

Subsequent studies in chickens from vaccinated mothers have found that CoxAbic can reduce oocyst output by 50-80% (Wallach, 2002) reduce lesion scores in challenged birds (Michael *et al.*, 2007) and produce similar effects to traditional coccidiostats on feed conversion rate, weight gain and mortality (Michael, 2003). In an independent study it was also found that chicks from CoxAbic treated mothers had significantly higher levels of circulating anti-*Eimeria* antibodies and that these chicks had lower lesion scores (Ziomko *et al.*, 2005). However, early pilot data suggests that a major part of the protective effect may be due to the adjuvant, and it's not clear if the specific immune response to the vaccine antigens is important.

Table 2.1: The effect of maternal vaccination with coccidial antigens on offspring ability to deal with a live challenge of three species of *Eimeria*. Data presented as from Table 1, Maternal immunity conferred by immunisation of breeding hens with affinity purified gametocyte antigen (APGA) (Wallach *et al.*, 1995b)

	Hatchling oocyst excretion (x 10^{-6}) (mean ± se) for egg collection period (challenge oocysts)					
Immunizing agent	**Days 28-39 (*E. maxima*)**	**Days 42-53 (*E. maxima*)**	**Days 56-67 (*E. maxima*)**	**Days 98-109 (*E. maxima*)**	**Days 98-109 (*E. acervulina*)**	**Days 112-123 (*E. tenella*)**
None	5.1 ± 0.7^{ab} (8)	10.9 ± 0.3^{a} (10)	10.8 ± 1.0^{a} (7)	9.9 ± 0.7^{a} (7)	53.9 ± 6.6^{a} (10)	ND
PBS in FCA	4.5 ± 1.3^{ab} (8)	7.1 ± 1.3^{b} (7)	8.7 ± 0.8^{b} (8)	3.7 ± 0.6^{b} (8)	37.2 ± 6.4^{bc} (11)	2.6 ± 0.6^{a} (10)
40 ug crude merozoite extract in FCA	4.1 ± 0.7^{ab} (8)	8.5 ± 1.0^{a} (9)	9.1 ± 1.1^{ab} (9)	ND	ND	ND
150 ug crude unsporulated oocyst extract in FCA[e]	ND	ND	ND	9.2^{a} (10)	59.7^{ab} (12)	3.9^{a} (8)
APGA (from 4 x 10^5 gametocytes) in FCA	2.9 ± 0.4^{ac} (6)	4.8 ± 0.6^{bc} (8)	8.7 ± 1.8^{ab} (8)	4.8 ± 0.9^{b} (7)	29.4 ± 5.7^{c} (11)	1.7 ± 0.4^{b} (10)
APGA (from 1 x 10^5 gametocytes) in FCA	5.5 ± 0.8^{b} (8)	4.1 ± 0.2^{cd} (7)	7.5 ± 1.2^{b} (7)	ND	ND	ND

Values in parentheses show number in group

ND, not done

[a,b,c,d] Within columns, those groups showing no superscripts in common indicate significant differences (p <0.05, one way ANOVA, Student's t-test)

[e] Mean counts of birds caged in groups

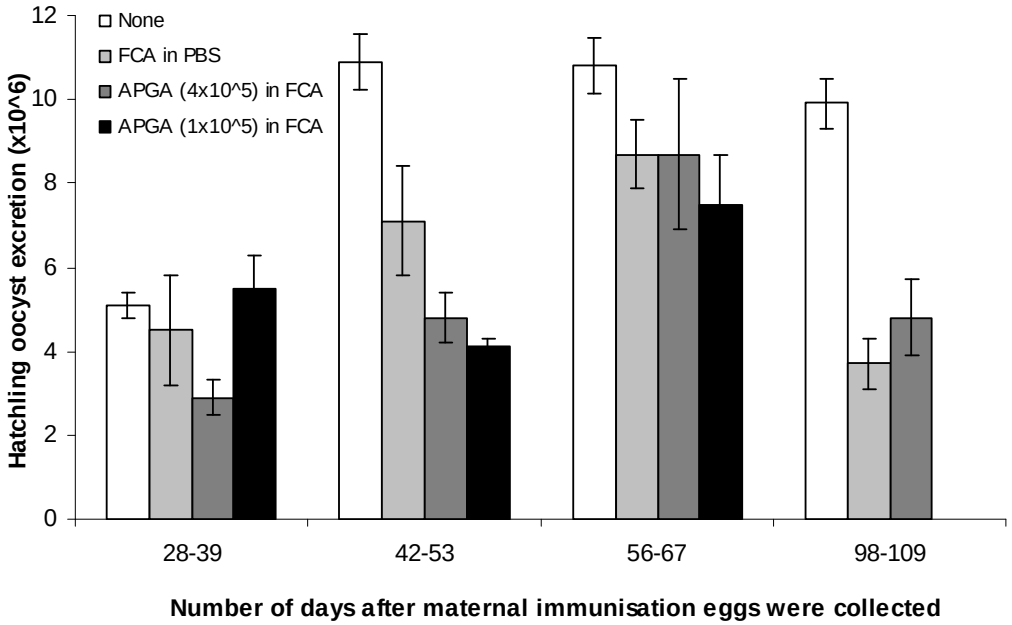

Figure 2.2: The effect of maternal vaccination with coccidial antigens on offspring ability to deal with a live challenge of *Eimeria maxima* as measured by oocyst excretion. Data presented as means (±se) as taken from Table 1 (Wallach *et al.*, 1995b). Only data from the vaccine components are included so as to aid clarity. FCA = Freund's Complete Adjuvant; PBS = Phosphate Buffered Saline; APGA = Affinity Purified Gametocyte Antigen – the antigens used in the CoxAbic vaccine.

The evidence so far suggests that CoxAbic, or the adjuvant administered with the vaccine, appears to be capable of producing an immunological response to coccidiosis and reducing the related pathologies in chickens. Furthermore, the subunit used in the vaccine is from a relatively conserved part of the *Eimeria* genome and has been shown to produce cross-species protection (Wallach, 2003). There is therefore considerable interest in whether it may be protective in gamebirds and offer an alternative control strategy to that of the soon to be phased out feed-based prophylactics. However, it is important to understand the costs and benefits associated with the maternal vaccination in both the mothers and offspring in order to conclusively evaluate the economic benefits.

When comparing the vaccine across avian systems it is important to consider differences in the husbandry practices within the systems that could potentially confound comparisons. A major potential confounding factor when comparing the game bird and poultry systems is that of cleanliness. The game bird system traditionally rears birds outside on pasture that has had many generations of birds on it. Wild hens caught annually and introduced on to the rearing field can import large numbers of parasites, potentially repopulating the pasture parasite population that may have diminished through the winter. However, continental game farmers often keep birds on raised wire cages during the laying period to reduce disease exposure and there is now a limited, but increasing trend for British game farmers to do the same. This is similar to the poultry industry where birds are reared in very clean environments, with regularly cleaned or disinfected litter. This is important when comparing a vaccine across the two systems as birds from each system will have a

different history of disease exposure with the pheasants potentially having a well established immunological response to specific pathogens, having been exposed to them throughout their lives and the chickens being relatively immunologically naïve as a consequence of the much cleaner rearing system.

In this chapter I test the novel coccidiosis vaccine CoxAbic (Wallach, 2001) to assess the positive and negative effects of vaccination in pheasants (*Phasianus colchicus*). Possible costs include the detrimental effects on the body condition of breeding females and any reduction in reproductive output. Specifically, I will examine the effect of different components of the CoxAbic vaccine on reproductive effort and body condition and then test whether vaccination confers any benefit to females when faced with challenge with a dose of live *Eimeria*. I then examine the effect of housing substrate on these traits and how this affects responses to vaccination.

2.2. The effects of CoxAbic and individual vaccine components on maternal traits

2.2.1. Materials and methods

To test the effects of CoxAbic and its associated adjuvant on the reproductive output and responses to infection of breeding female's three treatment groups were established in the field season of 2006. These were a control group (treated with PBS), an adjuvant only group (treated with Freund's incomplete adjuvant) and a vaccinated group (treated with the CoxAbic vaccine which contained APGA and FIA). Seventy two females were randomly selected and divided equally between

three large communal pens prior to treatment. However, before treatment could be administered the birds were released by animal rights activists, leaving only twenty seven females. The remaining females were housed together in one large pen but randomly allocated between treatment groups. Males were housed communally until needed for mating. All birds were marked with uniquely numbered leg rings and all birds were weighed (to the nearest gram) and morphometrics taken (tarsus to the nearest mm). A one way ANOVA was performed using baseline body condition index (calculated as the residual index) as the dependant variable to ensure no difference existed between the groups by chance ($F_{2,24} = 0.01$, $p = 0.913$). All birds were housed on concrete and the concrete was disinfected weekly with Virkon S solution (Dupont Animal Health Solutions) and gluteraldehyde to minimise parasite levels.

Prior to treatment a baseline blood sample (150 μL) was taken from the vena brachialis. These early samples were in order to account for any prior pathogenic exposure and to provide a baseline pre-immunisation response. These blood samples were then centrifuged (for 5 minutes at 900 rpm) and the supernatant (serum) removed and stored at -20^{o}C in 1.5 mL microtubes for later IgY analysis (Chapter 4). Blood samples were taken weekly until the end of the experimental period.

Females were housed communally in a 20’ x 30’ pen made of six interconnected 10’ x 10’ compartments. Each 10’ x 10’ compartment had one overhead nipple drinker, one 20 L feeder, a grit tray, a plastic laying box and two areas of *Cupressocyparis leylandii* brashing for cover. Males and females were fed Pheasant Breeder Pellets

(Gamekeepa Feeds; 28% protein). After one week of acclimatisation the females were treated with 0.5 mL of their assigned treatment in the pectoral muscle, with a second dose administered four weeks later. Immediately after the second vaccination females were divided between three 10' x 20' pens with three females from each of the three treatment groups per pen to give a total of nine females per pen. A male was introduced into each pen and allowed to mate for one week. After this time the males were returned to their communal pen and females were placed in individual 5' x 10' laying pens with a plastic laying box, an area of brashing to hide in during husbandry interventions, a sharp sand "nest" and *ad libitum* food and water. Eggs were collected daily and cleaned, weighed (to the nearest 0.01 g) and measured (length and breadth) to the nearest 0.1mm.

Egg volume (V) was calculated using a species specific calculation (Hoyt, 1979) based on linear dimensions (L = length, B = maximum diameter) and a species-specific volume coefficient (K_v; $K_{v.Phasianus\ colchicus} = 0.497$) as shown below:

$$V = K_v \cdot LB$$

Total reproductive effort was calculated as the total volume of eggs laid during the period that hens were in the laying pens. Body condition index was analysed as well as body weight to examine any trade-off between condition (musculature and fat deposits) and actual body size. There are a number of techniques for measuring condition ranging from a simple and subjective assessment of musculature around the breastbone (Gregory and Robins, 1998) to more complicated indices (Reviewed

by Jakob *et al.*, 1996). Throughout this study body condition was calculated using the residual index which is calculated by plotting a regression of body weight against a linear body measurement such as their tarsus length, and then using the individual's residual from the fitted line as their condition index. This therefore gives an indication of the individual's condition relative to the population average. This index is preferable over the simpler ratio indices which divide the mass by a linear measure, as these indices often do not properly control for linear size resulting in individuals with larger linear measurements having relatively larger condition scores. This is especially true in pheasants where weight in a healthy hen can range from 800g to 1300g whereas tarsus length varies from 40-50mm. Hen weight was measured at the beginning and end of the 2006 season.

2.2.2. Statistical analyses

The effect of treatment on egg characters was examined with a linear mixed model with hen treated as the independent unit throughout. Egg volume, egg weight, clutch size or total reproductive effort were entered as dependant variables. Treatment was entered as a fixed effect with clutch size included as an additional covariate when examining the effects of treatment on egg volume or weight. Maternal ID was included as a random effect. The effect of treatment on change in hen weight or condition was examined using the change in weight or condition from the beginning to the end of the experimental period as the dependant variable. Treatment was included as a fixed effect. Results are presented from the minimal models.

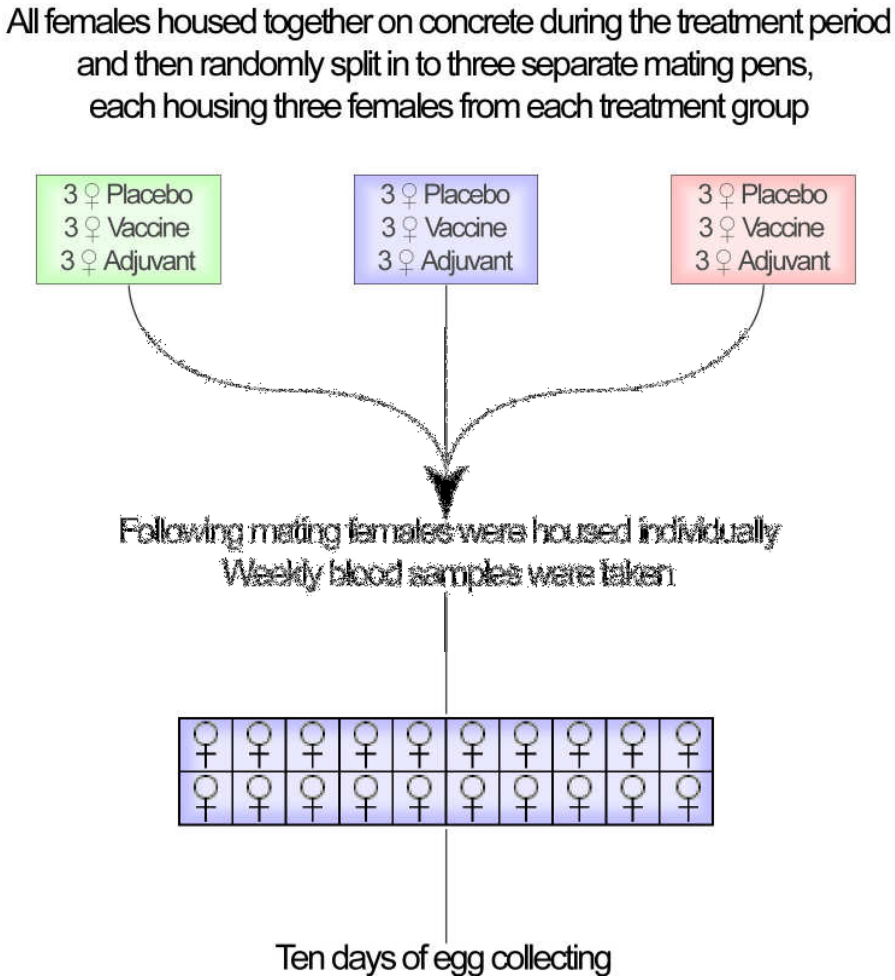

Figure 2.3: Experimental schematic for 2006

2.2.3. Results

2.2.3.a. The effect of treatment on egg characters

There was no evidence for any effect of treatment on egg volume ($\chi^2_{2,323} = 3.26$, $p = 0.196$), egg weight ($\chi^2_{2,323} = 0.89$, $p = 0.642$), clutch size ($\chi^2_{2,24} = 1.76$, $p = 0.415$) or total reproductive effort ($\chi^2_{2,24} = 3.28$, $p = 0.194$).

2.2.3.b. The effect of treatment on hen weight and condition

There was no evidence for any effect of treatment on change in weight ($\chi^2_{2,25} = 0.06$, $p = 0.971$) or condition ($\chi^2_{2,25} = 0.18$, $p = 0.673$) throughout the experimental period or on final weight ($\chi^2_{2,25} = 1.77$, $p = 0.413$) or condition ($\chi^2_{2,25} = 0.14$, $p = 0.930$).

2.3. The effects of CoxAbic and housing substrate on maternal traits

*2.*3.1. Materials and methods

No difference was detected in 2006 between the treatment groups, and so it was decided to remove the FIA treatment group to provide more power to explore the effects of the vaccine relative to the placebo. Due to the slow loss of IgY seen throughout the season (See Chapter 4), and its presumed link to lack of environmental exposure to coccidia it was also decided to add a housing substrate treatment in 2007. This was to allow us to see if the protection offered by CoxAbic is better than the protection offered from environmental challenge alone, or if CoxAbic only offers protection when environmental exposure is removed (on concrete). This

is also useful for the industry, especially when comparing with France where it is still common practise to house birds in wire floored cages during the laying period. This is comparable to the concrete substrate we used as the substrate was kept clean and so exposure to coccidia will have been minimal.

Eighty female and 8 male birds were randomly selected from overwintering stock. Females were then divided between two replicates of forty, staggered two weeks apart. Within these treatments birds were housed on either grass or concrete to allow comparison between normal rearing conditions (grass), where birds inevitably encounter low levels of a wide range of environmental pathogens (including coccidiosis), and a clean environment (concrete) where there should be little environmental exposure to any pathogens.

In the first replicate, forty females were randomly divided between treatment groups; with twenty receiving CoxAbic and twenty receiving PBS to act as an inert control. These groups were then divided between rearing substrate; each treatment group was housed in two pens on each substrate with five birds treated with PBS and five with CoxAbic in order to control for housing effects. A second booster vaccination was given four weeks later.

Before treatment morphometrics, weight and condition score were compared between treatment groups using a two-way ANOVA to ensure no differences existed between groups by chance. However, there was a non-significant trend for hens to be raised on grass to be in better condition ($F_{1,77} = 3.83$, $p = 0.051$). This arose from

using a simple ratio index rather than the more effective residual index that was adopted later in the study. This was therefore controlled for statistically in subsequent analyses (for further details see methods). A small nest like area of sharp sand was provided to encourage nesting and to reduce cracked eggs but this was often ignored by the birds. Concrete pens were washed and swept clean once a week as with the 2006 field season. Pen sizes and pen furniture were also the same as 2006.

Immediately after the second vaccination females were divided between one of 2 pens on their designated housing substrate, with 5 birds from each of the two treatment groups present in each (10 birds per pen), and one of 4 males (to accommodate a 1:10 male to female ratio) to allow fertilisation to occur. Females were allowed access to the male for a week and were then moved and housed in individual laying pens on the same housing substrate as described for 2006.

Once moved back to communal pens, weekly weights and blood samples were continued for a further five weeks. Then in order to test the effectiveness of the CoxAbic vaccine in protecting mothers from the effects of *Eimeria* females were challenged with a dose of 50 live sporulated oocysts. The effect of this challenge was monitored by weekly weight and condition measures, egg characteristics such as volume and weight and survival. Monitoring continued for two weeks until the end of the experimental period, which allowed for 7-9 days of observation after the peak of oocyst output 5-7 days after infection (Liou *et al.*, 2001).

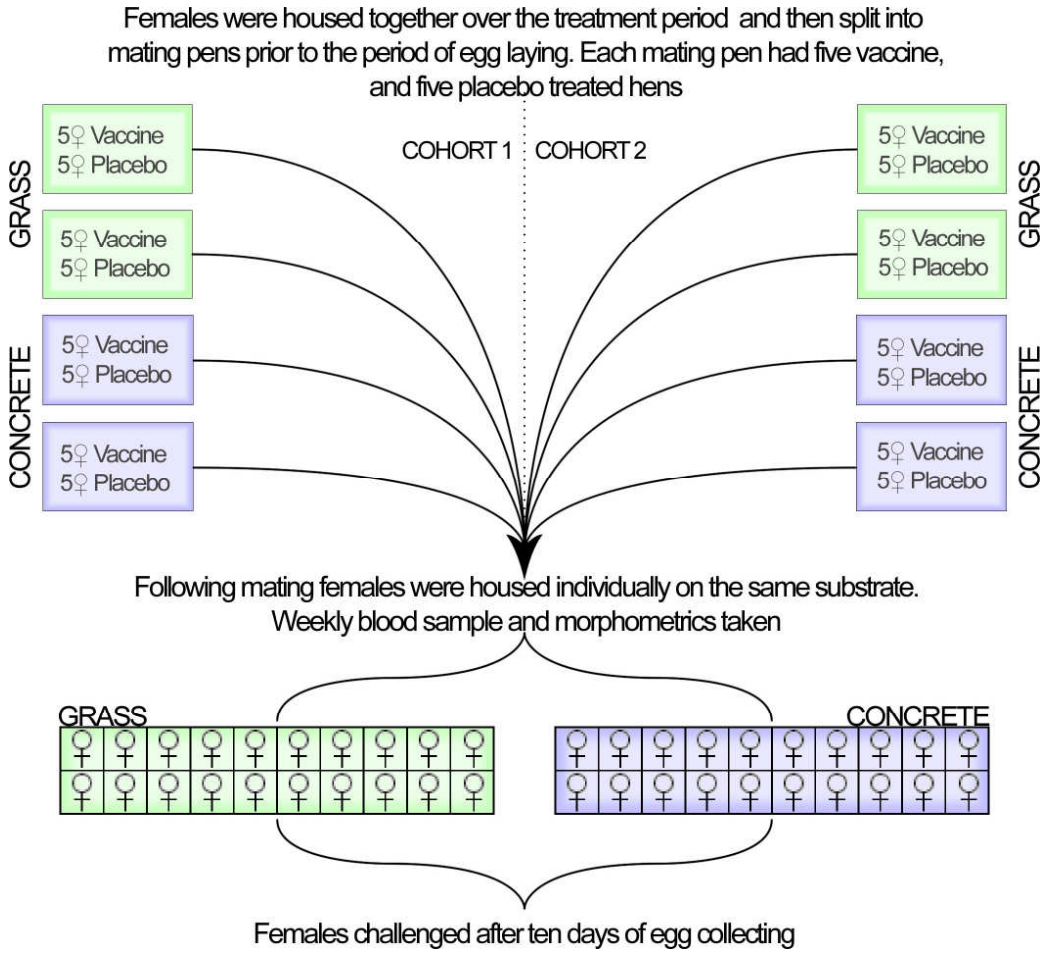

Figure 2.4: Experimental schematic for 2007

The laying season for pheasants starts in late March or early April and stretches until the middle of July. During the peak of the laying season (May-June) hens lay an egg a day. This period is energetically stressful, requiring 21-30% of daily energy intake to maintain body condition (King, 1973), and so any extra stresses caused by environmental conditions, predation or immune challenge could potentially lead to alterations in egg characteristics.. All data on egg characteristics are collected from the two week laying period, as this is the only time it is possible to assign eggs to females. Because of the energetic stresses of this period females also lose weight and body condition and so it should therefore be possible to examine the weight losses between treatment groups to see if there is any additional cost of treatment reflected in weight or condition.

The experiment was then replicated with a second cohort of 40 females two weeks after the first cohort.

2.3.2. Statistical analyses

A mixed model approach was used, with the hen treated as the independent unit in all analyses. All variables were analysed using residual maximum likelihood (REML) analysis with treatment, substrate (and treatment by substrate interaction), and (due to it only having two factors) cohort as fixed model effects and maternal ID as a random effect. The cohort effect is not strictly repeatable, and so should more likely be entered as a random effect in these models. However, as there are only two factor levels it was decided instead to include it as a fixed effect. Furthermore, many of the models described explored the effect of including cohort as a fixed or random effect

and found no difference. Initial models included paternal ID as an additional fixed effect, but this had no effect and so was not included in subsequent analyses. Maximal models were explored from the top down, however independent effects of substrate and treatment were also examined. Data presented are from minimal models. In analyses of time series data, time was included as a continuous variable and tested for sphericity using the Mauchly criterion. Where this was found to be significant (and the assumptions of sphericity were not met) the model degrees of freedom were corrected with the Greenhouse-Geisser epsilon correction and a new *p* value calculated. Where the same data were examined using multiple tests the alpha value was corrected by using a Bonferroni correction. This is a simple, and relatively conservative method, and also does not depend on independence of the tests conducted on the data, and so better suits the models discussed below.

The effects of maternal treatment were examined by looking at egg production measures and hen weight and condition changes between treatment groups. Models looking at the effects of treatment and substrate included egg weight, egg volume, number of eggs laid, total reproductive effort (TRE = number of eggs x average egg volume), weekly weight (before challenge) and weekly body condition index (before challenge) as dependant variables. Models exploring egg volume and egg weight included egg number as a covariate to examine trade-offs between egg size and number. Birds that were recorded as laying zero eggs were removed from analyses examining reproductive effort because these birds were often actually laying eggs, but eating or destroying them before they could be counted. These birds (n = 6) were

not significantly biased toward treatment (CoxAbic n =4, PBS n = 2) or substrate groups (Grass n = 2, Concrete n = 4).

To maximise the power to detect an effect of CoxAbic on female traits the relevant parts of the data from the two experiments were also combined and reanalysed as above with year as an additional fixed effect.

2.3.3 Results

2.3.3.a. The effects of treatment and substrate on egg characters

Neither treatment ($\chi^2_{1, 655} = 0.01$, $p = 0.924$), substrate ($\chi^2_{1, 659} = 0.37$, $p = 0.544$), their interaction ($\chi^2_{1, 655} = 0.33$, $p = 0.568$) or cohort ($\chi^2_{1, 657} = 0.08$, $p = 0.775$) had any effect on egg volume or egg weight (treatment ($\chi^2_{1, 655} = 0.04$, $p = 0.844$), substrate ($\chi^2_{1, 659} = 1.22$, $p = 0.270$), their interaction ($\chi^2_{1, 655} = 0.33$, $p = 0.565$) and cohort ($\chi^2_{1, 657} = 0.45$, $p = 0.504$)).

The effects on clutch size were examined next. This found that hens reared on grass ($\chi^2_{1, 71} = 18.94$, $p = < 0.001$; Figure 2.5), or laying earlier in the year ($\chi^2_{1, 71} = 20.68$, $p = < 0.001$) produced more eggs. However, neither treatment ($\chi^2_{1, 69} = 0.14$, $p = 0.710$) nor the substrate by treatment interaction ($\chi^2_{1, 69} = < 0.01$, $p = 0.948$) had any effect on clutch size.

This meant that that hens reared on grass ($\chi^2_{1, 71} = 18.82$, $p = < 0.001$; Figure 2.5), or laying earlier in the year ($\chi^2_{1, 71} = 19.60$, $p = < 0.001$) invested more in total

reproductive effort. Whereas treatment ($\chi^2_{1, 69} = 0.12$, $p = 0.731$) and the treatment by substrate interaction ($\chi^2_{1, 69} = 0.03$, $p = 0.860$) had no effect. (A Bonferroni correction was used to correct the alpha level to 0.025 to account for the two methods of examining this data.)

Analysis of the combined data from 2006 and 2007 placebo and vaccinated groups with year treated as a fixed effect confirmed these results. Treatment ($\chi^2_{1, 493} = 0.76$, $p = 0.384$), year ($\chi^2_{1,493} = 1.67$, $p = 0.197$) and cohort ($\chi^2_{1,492} = 0.46$, $p = 0.499$) had no effect on egg volume or egg weight (Treatment ($\chi^2_{1, 493} = 0.83$, $p = 0.362$), year ($\chi^2_{1, 493} = 0.83$, $p = 0.361$) and cohort ($\chi^2_{1,492} = 0.04$, $p = 0.849$)).

The effect of substrate on total reproductive effort can be further explored by including body condition at week 7 as an additional fixed effect. This model finds similar effects to those already stated, (no effect of treatment ($\chi^2_{1,69} = 0.08$, $p = 0.782$) or the treatment by substrate interaction ($\chi^2_{1,69} = 0.09$, $p = 0.759$) but significant substrate ($\chi^2_{1,70} = 13.38$, $p = < 0.001$) and cohort ($\chi^2_{1,71} = 21.86$, $p = < 0.001$) effects) with the addition of a significant positive effect of body condition at week 7 ($\chi^2_{1,70} = 7.89$, $p = 0.005$) and a significant interaction between substrate and condition at week 7 ($\chi^2_{1,70} = 6.91$, $p = 0.009$) such that females in poor condition and housed on concrete had the lowest total reproductive effort (Figure 2.6).

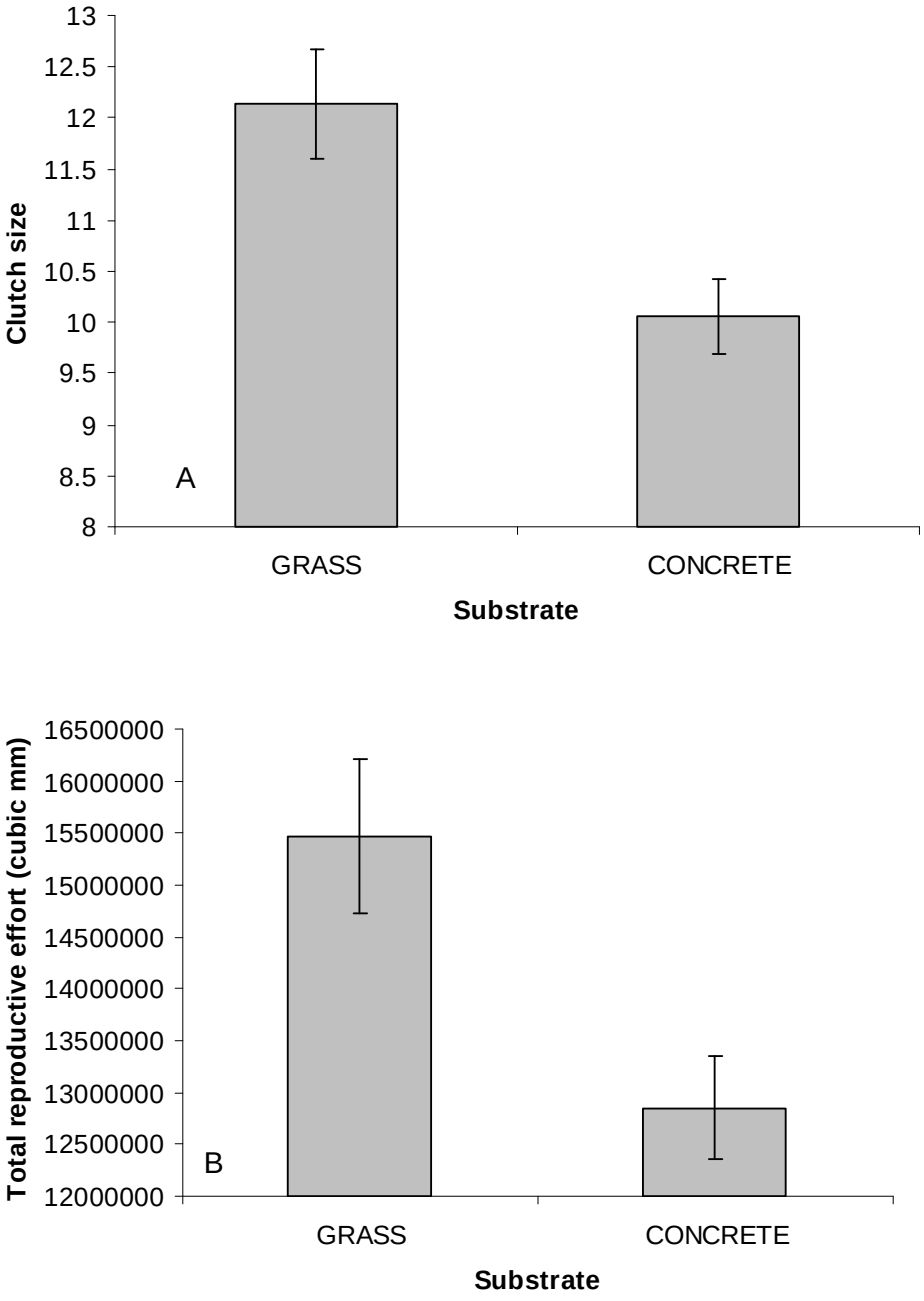

Figure 2.5: The effect of substrate on mean clutch size (±se) (A) and total reproductive effort (±se) (B), estimated from the minimal model

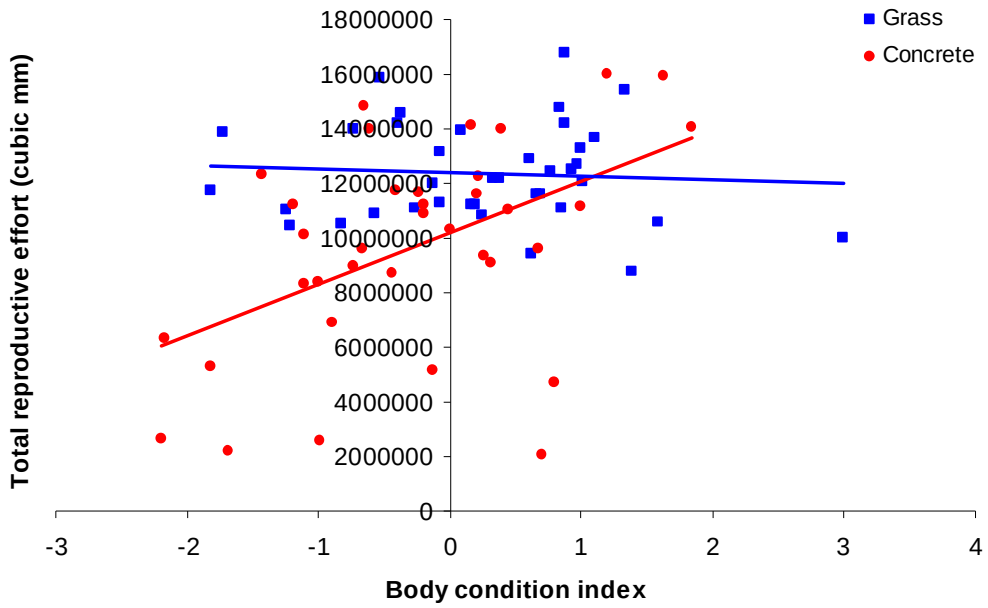

Figure 2.6: The effect of body condition index and substrate during laying on total reproductive effort for hens in 2007 with trend lines.

2.3.3.c. The effect of treatment on hen weight and condition

The effect of treatment on body condition and weight was examined by looking at weight and body condition change post -treatment but prior to challenge (week 13) with a linear mixed model. A repeated measures mixed model was used to explore the change in weight from week four to the week of challenge (Figure 2.7). Data did not fit the assumptions of sphericity and so were corrected with the calculated Greenhouse-Greisser epsilon value (0.66). Substrate had a significant effect on the change in weight from week 4 to week 13 with hens on grass losing significantly less weight ($\chi^2_{0.66,\ 526.7} = 6.55$, $p = 0.011$). However, treatment ($\chi^2_{0.66,\ 524} = 1.20$, $p = 0.274$), the substrate by treatment interaction ($\chi^2_{0.66,\ 524} = < 0.01$, $p = 1.000$) and cohort ($\chi^2_{0.66,\ 525.4} = 1.74$, $p = 0.188$) had no effect on the change in weight.

A repeated measures linear mixed model was used to explore the change in body condition index from one week after final treatment to the week of challenge. Substrate was found to have a significant effect on the change in body condition index (Figure 2.8), with hens on grass losing less condition ($\chi^2_{0.65,\ 518.7} = 8.90$, $p = 0.003$). However, there was no evidence that treatment ($\chi^2_{0.65,\ 518.1} = 0.61$, $p = 0.435$), the substrate by treatment interaction ($\chi^2_{0.65,\ 516.8} = 0.16$, $p = 0.689$) or cohort ($\chi^2_{0.65,\ 516.8} = 0.35$, $p = 0.554$) had any effect on change in body condition index.

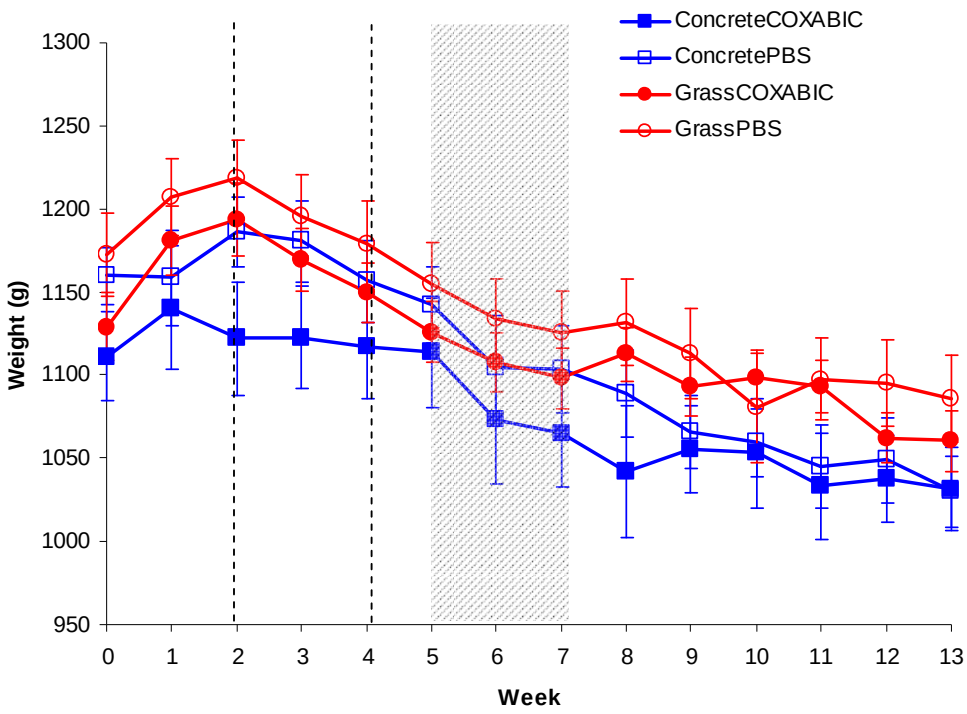

Figure 2.7: The effect of treatment and substrate on mean weekly weight (±se). Dotted lines represent vaccinations and half hatched areas represent the laying period.

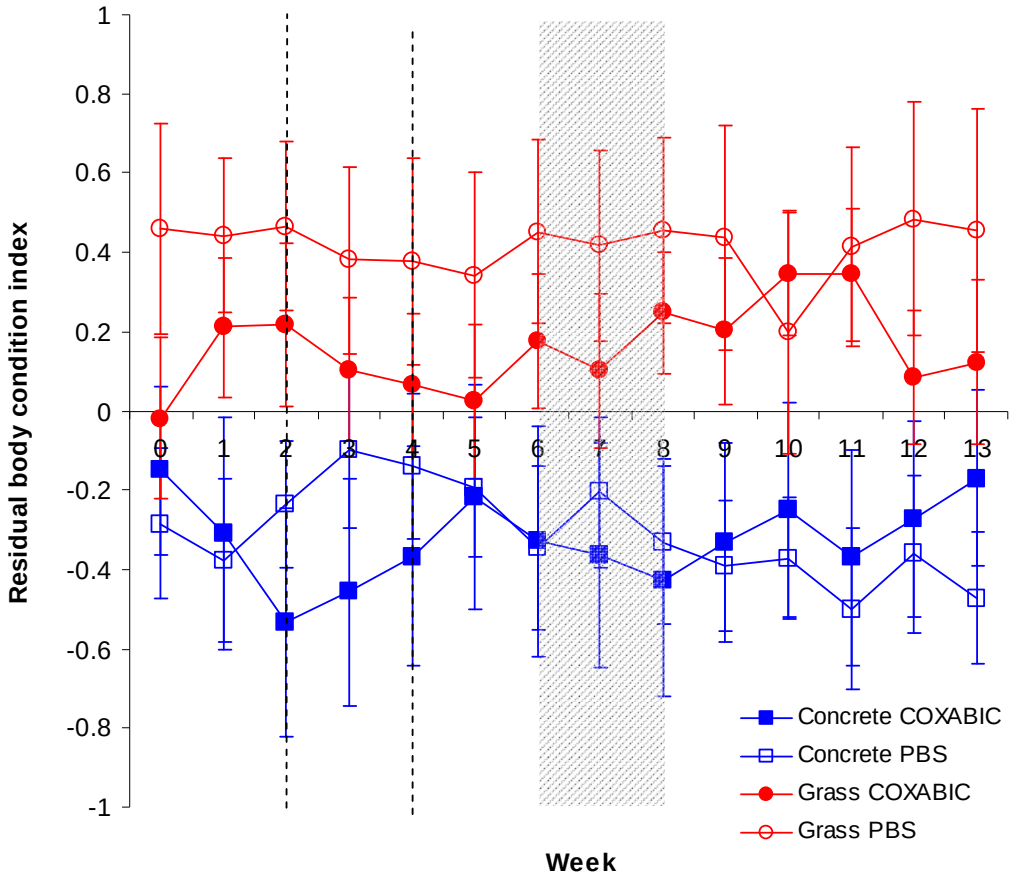

Figure 2.8: The effect of treatment and substrate on average weekly BCI (±se). Dotted lines represent vaccinations and half hatched areas represent the laying period.

2.3.3.d. The effect of vaccination on hens ability to cope with live challenge

In the 2007 experiment all females were challenged with 50 live oocysts at week 13 and then monitored for a further two weeks to assess any benefits of vaccination in terms of reduced mortality or reduced weight and loss of condition. Previous work on *Eimeria* in pheasants has shown that oocyst output can peak 5-7 days after inoculation (Liou *et al.*, 2001) which corresponds with a peak in intestinal damage and therefore loss of weight or condition should be visible soon after this time. This was analysed using the change in weight or condition from week 13 to week 15 as the dependant variable in a linear mixed model with substrate, treatment, their interaction and cohort as fixed effects. There was no evidence for a significant effect of treatment ($\chi^2_{1, 59} = < 0.01$, $p = 0.998$), substrate ($\chi^2_{1, 61} = 0.51$, $p = 0.474$) or the substrate by treatment interaction ($\chi^2_{1, 59} = 0.57$, $p = 0.449$) on weight loss. Furthermore, there was also no effect of treatment ($\chi^2_{1, 59} = 0.02$, $p = 0.892$), substrate ($\chi^2_{1, 61} = 0.86$, $p = 0.355$) or the substrate by treatment interaction ($\chi^2_{1, 59} = 0.56$, $p = 0.456$) on the change in body condition index. However, cohort was found to have a significant effect on the change in body condition index and weight with hens in cohort 2 losing significantly less weight ($\chi^2_{1, 62} = 17.76$, $p = < 0.001$) and condition ($\chi^2_{1, 62} = 13.92$, $p = < 0.001$).

Total mortality following live challenge was low with a small number of deaths per group (Concrete = 3, Grass = 2, PBS = 3, CoxAbic = 2).

2.4 Discussion

This chapter found no measurable effect of the CoxAbic vaccine, or Freund's incomplete adjuvant on any of the measured characters. However, substrate had a number of significant effects on egg characters and morphometrics with birds on grass producing larger clutches, investing more in total reproductive effort, and showing less weight and condition loss before challenge. Birds that bred later in the season (Cohort 2) or bred in 2007 were found to have reduced investment in egg production, and later breeding birds were found to have lost less weight or condition following challenge.

There was no evidence for any effect of CoxAbic of FIA on any of the measured characters. This could be because there was no effect of the vaccine on these traits or because any effect was equivalent to the cost of fighting low level environmental infection that was likely to be present in PBS treated birds. It could also be possible that whilst vaccination with a subunit removes problems associated with potential virulence and reversion, it does not produce the site specific but broad response of a live attenuated vaccine potentially leading to lower responsiveness to live challenge. However, the lack of evidence for a treatment effect on egg characteristics backs up previous work on the CoxAbic vaccine in chickens (Ziomko *et al.*, 2005) where treatment was found to have no effect on the number of eggs laid or their fertility and hatchability. The lack of a treatment effect was seen in both years, with different vaccine batches. This suggests it is a robust finding and is unlikely to be a consequence of a faulty vaccine batch or damage to the vaccine. Ideally however, the vaccine would have been tested with a further control group. This would have been a

small population of chickens treated with the CoxAbic vaccine and challenged with coccidia to ensure the vaccine was capable of providing protection. However, as a commercially produced and trialled subunit vaccine this should not have been necessary, since unlike a live vaccine, it is highly unlikely to have suffered any degradation during transit. Furthermore the vaccine was subject to this type of in-house quality control before being released.

It is also possible that the lack of evidence for a vaccine effect was due to a small sample size. Post-hoc power analysis of many of the models in this chapter showed relatively low (8-30%) power and suggested in excess of 2500 pheasants would be needed to detect an effect with the effect size found under these conditions. This study included 27 birds in the 2006 experiments and 80 in the 2007 experiments, compared with 4-7 birds per treatment group in Wallach's original experiments. Therefore it would be expected that our work would have sufficient power to detect effects of a similar size. However, it is possible that the vaccine effect was much smaller in pheasants and so would require a larger sample size to see the effect. Given the effect size in these experiments post-hoc power analysis shows a sample size of over 2500 pheasants would be needed to provide 80% power to detect an effect. The possibility of reduced reaction to the vaccine in pheasants seems unlikely given the conserved nature of the vaccine antigens, but if this is the case it raises concerns about the efficacy of the vaccine, such that if the vaccine produced a relatively lower response that therefore needs a larger sample size to elucidate it, then it is perhaps likely that the vaccine is unable to produce a strong enough response to offer any significant protection against infection.

The effect of substrate at first seems to be contrary to expected hypotheses because it has previously been assumed that housing birds in a cleaner environment such as concrete should reduce their exposure to pathogens and so they should be able to invest more in maintenance and egg production. However, there are a number of potential factors contributing to the contrary. For example, birds on grass may have been able to supplement their feeding with invertebrates and grass seeds. These are both important sources of protein which are important in maintaining weight and condition, and could have important effects on reproductive investment. Furthermore the interaction effect between condition and substrate suggests that the effect of body condition index on total reproductive effort is more important when housed on concrete, possibly due to the limiting effect this has on protein intake.

Protein has also been shown to be important in the immune responses of birds (Lochmiller *et al.*, 1993; Klasing, 1998; Kidd, 2004; Smith *et al.*, 2007). It has been shown that chickens can deposit 1-25 mg immunoglobulin per 1 g of yolk (Kitaguchi *et al.*, 2008) which can correspond to 10-20% of the females daily serum immunoglobulin levels (Kowalczyk *et al.*, 1985). Extra protein can therefore potentially help alleviate any costs associated with the nutritional demands associated with producing an immune response.

As well as supplying an extra source of protein the presence of insects and grass and the act of foraging for them is likely to have helped to enrich the bird's environment, which has been shown to have positive effects on behaviour, physical wellbeing and

a supposed effect on stress (Craig and Adams, 1984; Dawkins, 1988; Rushen and Depassille, 1992). A particularly important source of enrichment for birds housed on grass rather than concrete is likely to be the ability to dust bathe. Dust bathing is important in many bird species, as demonstrated by the attempt to dust bathe even in the absence of a suitable substrate (Lindberg and Nicol, 1997). The inability to dust bathe can have important effects on the behaviour of the birds (Vanliere and Wiepkema, 1992) potentially leading to an increase in stress hormones like corticosterone (Vestergaard *et al.*, 1997) and stress related behaviours such as feather pecking (Vestergaard *et al.*, 1993; Huber-Eicher and Wechsler, 1997) which can also impact on the stress of nearby individuals.

The concrete substrate was also more susceptible to changes in the weather and quickly overheated, flooded, or chilled as the weather changed. The grass environment on the other hand maintained a steadier environment potentially resulting in reduced environmental stress compared to the birds on concrete. The combination of a number of these factors, or stress alone in the birds on concrete when compared to those on grass could in turn have affected their body condition and reproductive output, for example through a corticosterone based mechanism (DeVries *et al.*, 1997; Sakami *et al.*, 2004; Morales *et al.*, 2006). Taken as a whole these substrate effects suggest that the benefit of reduced exposure to pathogens when on concrete is relatively small compared to the mainly stress induced cost associated with being housed on concrete, due to the lack of both dietary and behavioural enrichment.

The effect of timing of breeding (cohort) previously mentioned seems to be due to the two week difference between the cohorts rather than any effect of husbandry. Given that the birds lost weight through the season and cohort 2 was two weeks after cohort 1 it makes sense that birds from cohort 2 will be lighter than those of cohort 1 at any given experimental time point (Figure 2.9). The weight loss itself is most probably caused by daily egg laying, and it is interesting to note that average weight increased from week 0 to week 2 which could reflect an investment in body condition specifically to buffer the expected losses throughout the laying season. This profile of weight gain followed by weight loss is similar to that seen in studies of American ring-necked pheasants, and seems to be especially typical of first year birds (Kirkpatrick, 1944; Kabat *et al.*, 1950; Gates and Woehler, 1968), this pattern has also been seen in many other bird species (Experimental data and review by Baldwin and Kendeigh, 1938).

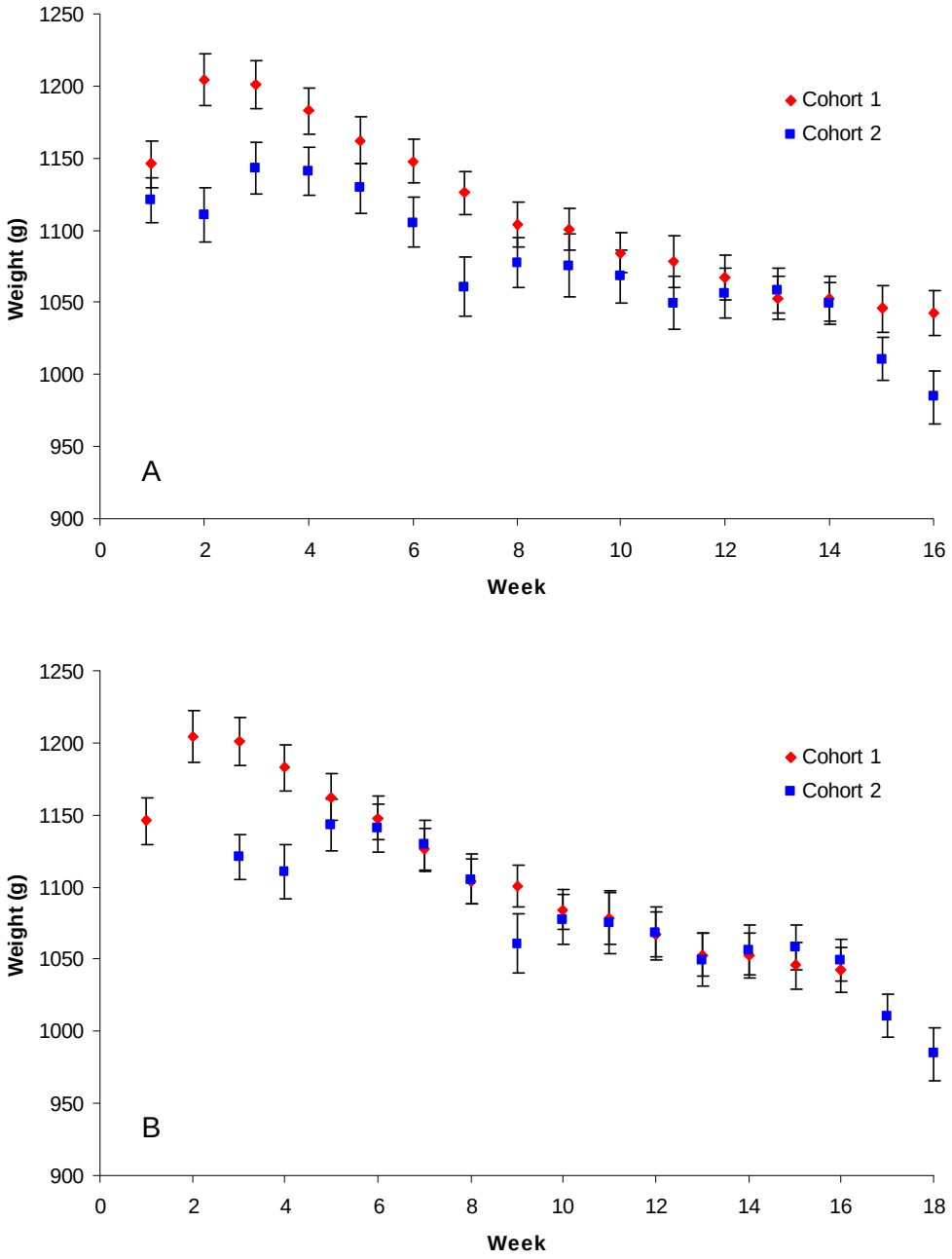

Figure 2.9: Average weight of each cohort through the season. Graph A shows the weekly data (±se) presented as analysed per experimental time point. Graph B shows the data as real time points (±se). Cohort 2 began two weeks after cohort 1 and this is reflected by the difference in start and end point shown in Graph B.

Birds bred in 2007 showed lower reproductive output (clutch size and total reproductive effort) than those in 2006, with an approximately 4% lower total reproductive effort. This is potentially due to differences in the weather as the 2006 laying season (May-July) had lower rainfall (2006, 167.8mm; 2007, 319.4mm) and more hours of sunshine (2006, 742.3h; 2007, 549h), both of which are important to laying birds.

Overall these results show that housing substrate can have significant effects on pheasant condition and reproduction. These effects should be explored more fully both in the presence and absence of environmental pathogens so that the industry can make a more informed decision on best rearing practice. This is especially relevant now due to the increasing dependence on pheasants imported from France, where it is normal to house hens in raised wire cages. Based on the evidence provided in this chapter it is possible that these imported birds will be in worse condition relative to a similar bird raised on grass, and that these effects may well be passed on to their offspring leading to possible effects on their long term survival and subsequent reproductive success.

Chapter 3 - Benefits of the CoxAbic vaccine to chicks

3.1 Introduction

As early as the 18th century Geert Reinders showed that cows that survived rinderpest infection produced calves that were also resistant (Oldenkamp, 1998). This is potentially the first documented example of maternally derived immunity, but it does not distinguish between genetic and maternal effects. For example, a genetically determined trait that improves the chances of surviving rinderpest or the transfer of maternal antibodies against rinderpest infection. Since then maternally derived immunity has been studied in a number of organisms including humans (Stetler *et al.*, 1986; Mooi and de Greeff, 2007; Sadeharju *et al.*, 2007), birds (Rose, 1972; Pihlaja *et al.*, 2006; Staszewski *et al.*, 2007; Gallizzi *et al.*, 2008), rodents (Adler and Foner, 1965; Good *et al.*, 2004), and even insects (Mousseau and Dingle, 1991; Fox and Mousseau, 1998; Sadd *et al.*, 2005).

The method of transfer varies with birds passing antibodies into the vitelline membrane surrounding the yolk (Rose, 1972; Tressler and Roth, 1987) and most mammals receiving antibodies via milk or colostrum (Adler and Foner, 1965; Israel *et al.*, 1995; Schnulle and Hurley, 2003; Sadeharju *et al.*, 2007), and in the case of carnivores, rodents and primates also across the placenta (Bruce-Chwatt, 1954; Story *et al.*, 1994; Takizawa *et al.*, 2005). The immune system of neonates is poorly developed and needs time to mature before it is able to competently protect the organism. However, neonates immediately encounter an environment full of potential pathogens and yet do not succumb to myriad infections. This is in part due

to the neonate's cell-mediated immune system which does not require such a long period of time to become effective, but also potentially due to maternally derived immunity which can provide some level of protection to the young whilst their own immune system develops. It is also important to consider that a neonate must balance the demands of a developing immune system against other factors such as growth and physical development (Merino *et al.*, 1999; Horak *et al.*, 2000; Merino *et al.*, 2000; Soler *et al.*, 2003; Mauck *et al.*, 2005; Pilorz *et al.*, 2005; Brzek and Konarzewski, 2007)

3.1.1. The benefits of maternal vaccination

Maternal immunity can be seen as providing a two-fold benefit to offspring. Firstly the transfer of immune components can protect them against disease and potentially increase survival (Heller *et al.*, 1990; Pihlaja *et al.*, 2006). Secondly the presence of these maternal immune components can block their ability to mount an immune response of their own, potentially forcing them to invest in other life history traits such as early growth rates, which may have a positive effect on subsequent offspring fitness (Keeler and van Noordwijk, 1993; Gebhardt-Henrich and Richner, 1998).

Maternal antibodies are important during early life as it can take up to several months for acquired immunity to fully develop (Apanius, 1998; Klasing and Leshchinsky, 1998), during which time offspring are dependant on maternal antibodies and their own slowly developing immune system for protection (Reviewed by Hasselquist and Nilsson, 2009). For example, maternally derived antibodies have been shown to help increase survival to 20 days in magpie chicks

(*Pica pica*) in the absence of specific experimental challenge, and to 45 days in chicken chicks (Heller *et al.*, 1990) challenged with *E. coli.*

Due to the blocking action of maternal antibodies, offspring may be prevented from investing in their own immunity, and due to being unable to mount an immune response, the cost of immunity may be reduced (Grindstaff, 2008), at least in the short term, potentially allowing investment in other life history traits such as early growth. Evidence for this may be seen in immunodeficient mothers, where the lack of transfer of immune components to the offspring may force the offspring to invest in immunocompetence at the cost of reduced investment in offspring growth leading to smaller offspring (Gustafsson *et al.*, 1994), though this may also be due to the cost of fighting infection, rather than of maintaining an immune system *per se*. This effect has been more clearly seen where an immune response is induced in the mother and offspring from challenged mothers show increased growth rates both in the presence of environmental challenge (Heeb *et al.*, 1998; Buechler *et al.*, 2002; Grindstaff, 2008) and absence of offspring challenge (Lozano and Ydenberg, 2002; Gallizzi *et al.*, 2008), potentially leading to longer term effects such as earlier fledging (Heeb *et al.*, 1998) or maturation (Kallio *et al.*, 2006). However, it is important to consider that an offspring's ability to respond to pathogens that the mother has previously encountered could have a significant genetic basis as well as being potentially due to the transfer of maternal immune components.

The effects of maternal immunity on offspring development are likely due to two related effects; reduction in the cost of mounting an immune response and reduction

in the cost of infection. For example, with circulating maternal antibodies there may be a reduced need to activate the potentially more costly innate immune system (Klasing and Leshchinsky, 1998; Råberg *et al.*, 2002), which can have negative effects on neonatal growth (Merino *et al.*, 2000; Mauck *et al.*, 2005). Furthermore, the presence of these maternal immune factors can help protect the offspring by providing short term immunity (Rose, 1972; Smith *et al.*, 1994b; Al-Natour *et al.*, 2004) thereby reducing the costs associated with infection, such as cellular and tissue damage and loss of energetic compounds required to mount an immune response.

Due to these potential benefits maternally derived immunity has received a lot of attention, both because of the combined potentials to protect neonates in areas of high disease risk, and because of the economics of vaccinating a few mothers who will pass on immunity to their many offspring. This is especially attractive to the livestock industry where mothers produce many offspring, and the offspring are subject to high levels of environmental pathogens.

One disease in which maternally derived immunity is potentially very useful is coccidiosis. Maternally derived immunity against coccidiosis was first demonstrated by Rose (1972), who showed that hens challenged with *Eimeria tenella* passed IgY (the avian equivalent of the mammalian IgG) through the yolk, and that this was protective to the chicks. This IgY could also be extracted from the yolk and injected *in ovo* into eggs from unchallenged mothers to give protection to their chicks. Previous work by Smith *et al*. (1994b) suggested that a large level infection of the mother (20 000 oocysts of *E. maxima*) could cause a 68-87% reduction in oocyst

excretion in the offspring when challenged with *E. maxima* or 41-62% reduction when challenged with *E. tenella* (depending on the time after maternal challenge that eggs were collected). This work, and other similar studies, has resulted in the production of the CoxAbic vaccine (Wallach, 2001). As this is a novel vaccine there is little data currently available. However, studies to date have shown it can be successful at reducing oocyst output by between 41.5% - 80% (Wallach *et al.*, 1995b; Wallach, 2002), as well as reducing lesion scores (a measure of intestinal damage) (Michael *et al.*, 2007), preventing reductions in feed conversion rate, limiting mortality (Michael, 2003) and increasing anti-*Eimeria* antibodies (Ziomko *et al.*, 2005). For a more detailed discussion of these studies see Chapter 2.1.3.

3.1.2. The costs of maternal vaccination

While it is clear there may be benefits to offspring of maternal immunity and therefore maternal vaccination, there may also be costs. For example, it has been repeatedly shown that maternally derived immunity can potentially interfere with the offspring's immune response in early life (Stetler *et al.*, 1986; Blomqvist *et al.*, 2003; Englund, 2007; Siegrist, 2007). In a human measles studies it has been shown that the inhibitory effects of maternally derived antibodies can last until about 12 months of age (as shown by IFN-gamma and IL-12 titres; Gans *et al.*, 1999), and in a rodent-malaria pups vaccinated with the component merozoite surface protein 1_{19} (MSP-1_{19}) vaccine showed a reduced ability to mount a humoral response for up to 2 weeks (Good *et al.*, 2004). This blocking effect has also been seen in chickens infected with controlled amounts of infectious bursal disease virus (Al-Natour *et al.*, 2004) and in a semi natural kittiwake (*Rissa tridactyla*) system using Newcastle disease virus

(Staszewski *et al.*, 2007). However, it is unknown whether this effect has a longer lasting impact on the offspring's immune system.

Chapters 1 and 2 have described the potential costs and benefits to the mother and offspring of maternally derived immunity and chapter 2 looked specifically at the costs and benefits to the mothers. This chapter will expand on this by looking at the benefits of maternal immunity to the chicks. This will be explored by looking at the effect of maternal substrate and treatment on chick growth and condition up to three weeks of age (before challenge) and chick growth, condition and oocyst output in response to a challenge with live oocysts from three weeks up to seven weeks of age.

3.2 Materials and methods

3.2.1. Experimental Design

The treatment groups established in experiment 2 described in chapter 2 provide the basis for this experiment and the hens mentioned below are those used in chapter 2. In summary, eighty hens were split into two cohorts and randomly and equally divided between the PBS and CoxAbic treatments and the grass and concrete housing substrates. After treatment females were split into groups of ten, with five females from each treatment group per pen. A male was introduced and allowed to mate for one week. Females were then moved to individual laying pens on the same substrate and eggs were collected daily for ten days. For further details refer to Chapter 2.

All eggs were collected, marked with maternal ID and weighed and measured. Any eggs with obvious defects (cracks, holes etc) were graded out at this point, but the remainder were stored in a cool, dark room and turned once daily until setting. On the day of setting all eggs were examined for defects and a maximum of 8 eggs per female were selected for incubation. Any eggs with cracked or damaged shells were discarded. There was no significant difference in the number of eggs set per treatment (CoxAbic = 281, PBS = 265; t = -0.36, df = 78, $p = 0.724$).

Eggs were split randomly and evenly between incubators to ensure a mix of treatments, substrates and mothers in order to account for incubator differences. Egg trays were weighed before and after eggs were placed in them to give a baseline egg weight. Eggs were incubated at standard temperature and humidity (37.5^{o}C, 55% RH) and turned every hour for 21 days.

At 3, 7 and 14 days egg trays were removed and weighed to ensure incubation was proceeding at the correct rate. Throughout incubation eggs lose water due to evaporation through the shell and internal metabolic processes. This water loss leads to the decreasing weight of the eggs. Experimentation at the Game and Wildlife Conservancy Trust has shown that eggs should lose 12-14% of their initial weight by day 21 of incubation, and that this loss should follow a linear curve. It is therefore possible to predict percentage weight losses and therefore predicted egg weights at any time during incubation. Any deviation from these predictions indicates improper humidity settings. For example, if weight loss is too high then humidity is too low and the eggs are losing too much water too quickly.

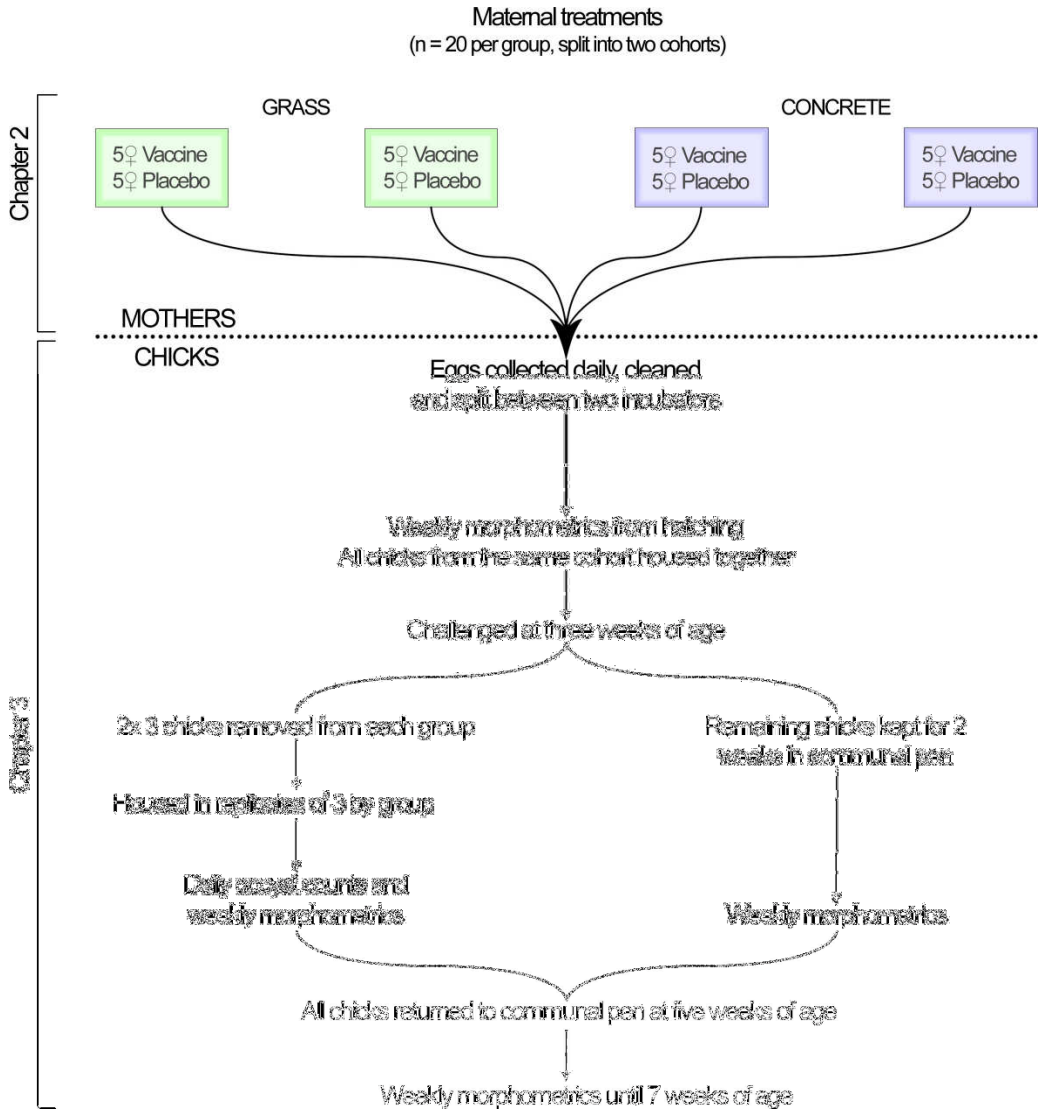

Figure 3.1: Experimental schematic

After 21 days all eggs were removed from the incubators and candled to check development. Any eggs that do not develop (due to damage or lack of fertility) show up with bright, empty shells and would have not hatched. At this point any small cracks in the eggs that were not visible on the day of setting were easy to see due to the damage to the underlying membrane. After the eggs were candled and graded three eggs from each mother were selected and placed in the hatcher in wire baskets according to maternal ID.

Eggs were left in the hatcher for two to three days with high levels of humidity. This kept the egg membranes soft and allowed the chicks to break through the shells. After two thirds of the eggs showed signs of pipping the incubator humidity was reduced to allow the chicks to dry out.

On hatching, chicks were weighed (to the nearest .01 grams), morphometrics (tarsus length) recorded and tagged with a unique coloured and numbered leg ring and patagial tag to allow identification of individuals, mothers, and maternal treatment group. Due to the high loss rate of leg rings in young chicks one identical ring was placed on each leg. Chicks were given *ad libitum* access to water in nipple drinkers (Quill Productions) and chick crumb (AB Agri Sportsman Feeds Range, 28.5% protein). Chicks were slowly changed to Game Grower No. 1 Pellets (23.5% protein) at 3 weeks of age and chick crumb was totally removed by 4 weeks of age. AB Agri Sportsman Feeds were used as this range uses organic ingredients that are free from any drugs (such as coccidiostats) that are routinely put in game feed and could potentially interfere with the experiments.

Following hatching chicks were caught once weekly to allow measurements of weight and tarsal length (to provide a growth curve for each individual). Measurements continued until the chicks reached seven weeks of age, at which time they would usually be moved to woodland release pens.

Chicks were housed together in 8' x 8' brooder houses with an attached 8'x8' night shelter and 10' x 60' outside run, as is standard rearing practice. For the first week chicks were confined to a 4' diameter round enclosure directly under the heat lamp, after which they were given full access to the brooder house. At 10 days the chicks were allowed into the night shelter, and by 14 days were allowed access to a 10'x10' portion of the outside run. By 20 days chicks were allowed full access to the outside run and the heat lamp was turned off during the day to begin the hardening off process.

In order to assess the effectiveness of the experimental treatments on chick health chicks were housed on concrete from time of hatching. At 3 weeks of age all chicks were challenged with a dose of 50 live sporulated oocysts. A subset of the offspring (n = 6 chicks per treatment, split between 2 pens to control for any pen effect) were moved to 5' x 10' pens raised up on wire. Faecal oocyst output in the subset of offspring was measured daily for two weeks. The weekly measurements of weight and morphometrics were used to test for an effect of treatment on response to a standard challenge of coccidia.

3.2.3. Statistical analyses

A mixed model approach was used, with the hen treated as the independent unit in all analyses. All variables except oocyst output were analysed using residual maximum likelihood (REML) analysis with maternal treatment, maternal substrate (and treatment by substrate interaction) and cohort as fixed model effects and maternal ID as a random effect. Average egg weight was included as a covariate. Repeated measures data were checked for sphericity using the Mauchly criterion. If the conditions for sphericity were not met then the model degrees of freedom and *p*-value were corrected with the calculated Greenhouse-Geisser epsilon value.

The benefits of maternal treatment were examined by looking at chick weight and body condition index (BCI). This was divided into four distinct time frames: hatching (week 0); pre challenge (weeks 0 - 3); post challenge (weeks 3-7); final (week 7). Growth rate was analysed from week 3 to week 7, during the period of linear growth.

There was insufficient data to use mixed models to analyse the benefits of maternal treatment on oocyst output, and so the means were analysed with a two way ANOVA.

3.3 Results

3.3.1. The effect of maternal treatment on chick growth and condition

Exploring the effect of maternal treatment up to three weeks of age (challenge) allowed for the examination of the direct effect of maternal immunity on growth in the absence of challenge. Firstly the effect of maternal treatment and maternal substrate on chick weight and condition at hatching was investigated. There was no evidence for any effect of treatment ($\chi^2_{1, 171} = 0.38$, $p = 0.535$), substrate ($\chi^2_{1, 175} = 3.43$, $p = 0.064$), or their interaction ($\chi^2_{1, 173} = < 0.01$, $p = 0.998$) on weight at hatching. Furthermore, cohort ($\chi^2_{1, 171} = < 0.01$, $p = 0.995$), and maternal condition ($\chi^2_{1, 171} = 0.08$, $p = 0.779$) also had no effect in the model or when explored separately. However, there was a significant positive effect of the egg weight on hatching weight ($\chi^2_{1, 175} = 97.14$, $p = < 0.001$). Previous analysis (see Chapter 2) showed no effect of treatment on egg weight.

The change in weight from hatching to week 3 was then looked at in the same way. Neither maternal treatment ($\chi^2_{1, 65} = 0.19$, $p = 0.664$), maternal substrate ($\chi^2_{1, 65} = < 0.01$, $p = 0.998$), their interaction ($\chi^2_{1, 65} = 0.29$, $p = 0.588$), or maternal condition ($\chi^2_{1, 67} = 0.10$, $p = 0.750$) had a significant effect on the change in weight (Figure 3.2). However, there was a significant effect of cohort, such that chicks that hatched two weeks later gained on average 10.291g (± 4.645) less ($\chi^2_{1, 69} = 4.31$, $p = 0.038$).

The effects of maternal treatment and maternal substrate on the change in body condition index and condition at hatching were also examined, with the effect of treatment and substrate on condition at hatching explored first. This found no

evidence for any effect of maternal treatment ($\chi^2_{1, 171} = 0.14$, $p = 0.705$), maternal substrate ($\chi^2_{1, 174} = 0.28$, $p = 0.594$), the substrate by treatment interaction ($\chi^2_{1, 171} = 0.44$, $p = 0.506$; Figure 3.2) or cohort ($\chi^2_{1, 171} = 0.04$, $p = 0.850$) on body condition at hatching, but there was a significant positive effect of maternal condition ($\chi^2_{1, 175} = 4.14$, $p = 0.042$) and egg weight ($\chi^2_{1, 175} = 64.08$, $p = < 0.001$) on hatchling condition. Previous analysis (see Chapter 2) showed no effect of treatment on maternal condition.

Finally the effect of maternal substrate and maternal treatment on change in body condition index from hatching to week 3 was examined. This found no significant evidence for any effect of maternal treatment ($\chi^2_{1, 64} = 0.29$, $p = 0.592$), maternal substrate ($\chi^2_{1, 66} = 0.11$, $p = 0.738$), their interaction ($\chi^2_{1, 64} = 0.02$, $p = 0.897$) or cohort ($\chi^2_{1, 67} = 1.03$, $p = 0.311$). However, maternal condition ($\chi^2_{1, 68} = 4.98$, $p = 0.026$) had a significant positive effect on the change in condition from hatching to week 3. Rerunning the model without maternal condition did not alter the significance of other terms in the model.

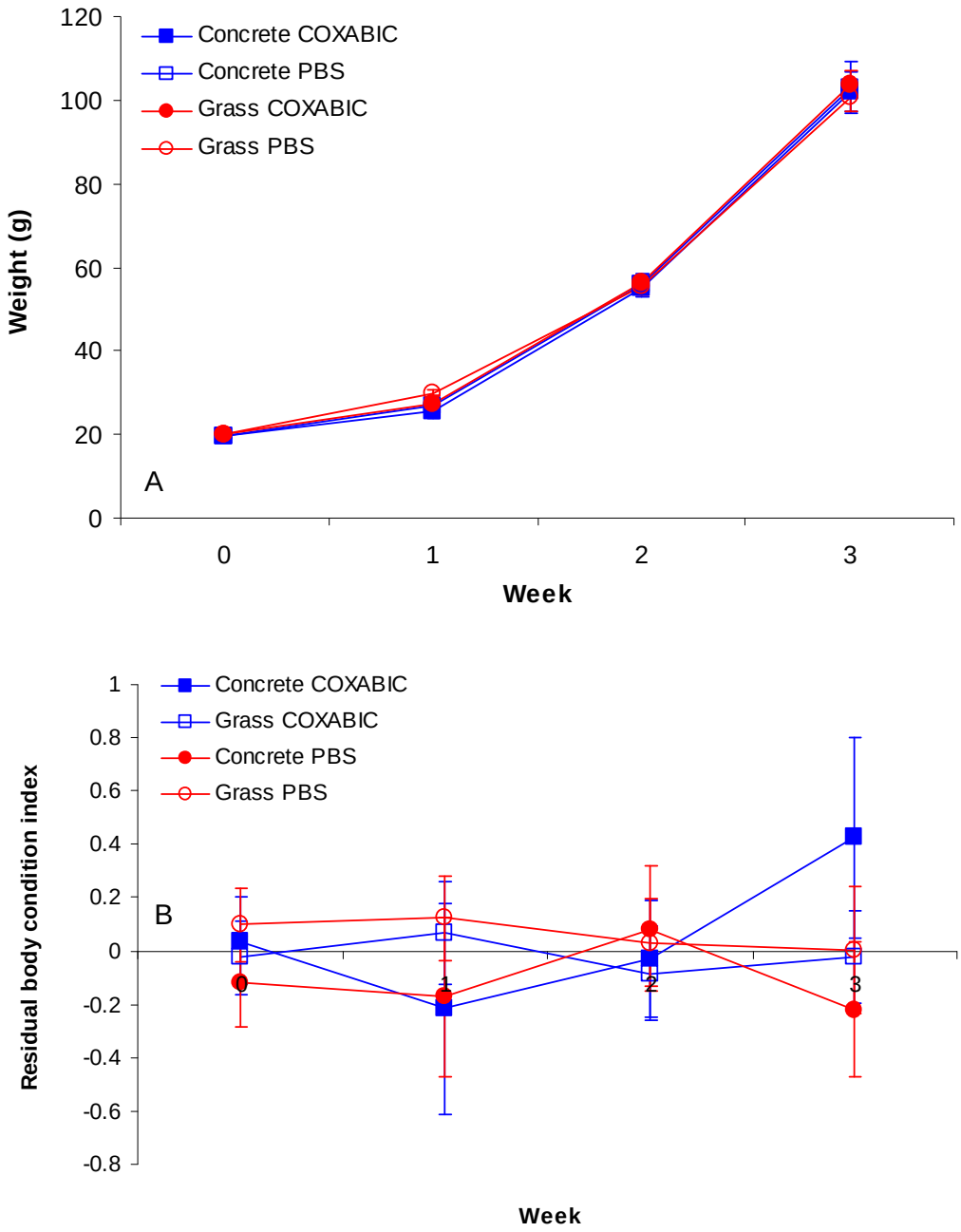

Figure 3.2: The effect of maternal substrate and maternal treatment on change in weight (±se) (A) and body condition index (±se) (B) from hatching to week 3

At three weeks of age all chicks were challenged with live coccidia and weight recorded until 7 weeks of age. Therefore from this point the experiment examined the ability of the vaccine to offer protection to offspring. There was no evidence for any effect of treatment ($\chi^2_{1,\ 260} = < 0.01$, $p = 0.989$), substrate ($\chi^2_{1,\ 263} = 0.80$, $p = 0.370$), their interaction ($\chi^2_{1,\ 260} = 0.07$, $p = 0.790$) or cohort ($\chi^2_{1,\ 262} = 0.41$, $p = 0.521$) on the change in weight from week 3 to week 7. This was also seen when exploring the change in body condition index from week 3 to week 7, as there was no evidence for any effect of treatment ($\chi^2_{1,\ 262} = 0.26$, $p = 0.612$), substrate ($\chi^2_{1,\ 260} = 0.01$, $p = 0.913$), the substrate by treatment interaction ($\chi^2_{1,\ 260} = 0.49$, $p = 0.484$) or cohort ($\chi^2_{1,\ 263} = 0.45$, $p = 0.504$).

Finally the effect of maternal substrate and maternal treatment on final weight and condition was examined. There was no evidence for any effect of maternal substrate ($\chi^2_{1,\ 29} = 0.05$, $p = 0.831$), maternal treatment ($\chi^2_{1,\ 29} = 0.71$, $p = 0.399$) or their interaction ($\chi^2_{1,\ 29} = 0.01$, $p = 0.938$). However, later hatched chicks were significantly heavier ($\chi^2_{1,\ 32} = 4.42$, $p = 0.035$) by an average of 60.46g (± 28.75). This was again repeated with body condition at week 7 as the dependant variable and found that chicks from hens raised on grass ($\chi^2_{1,\ 31} = 4.86$, $p = 0.028$) and hatched two weeks later ($\chi^2_{1,\ 31} = 4.95$, $p = 0.026$) were in better condition. However, there was no evidence for an effect of maternal treatment ($\chi^2_{1,\ 29} = 0.34$, $p = 0.559$) or the substrate by treatment interaction ($\chi^2_{1,\ 29} = 2.34$, $p = 0.126$)

3.3.2. The effect of maternal treatment on offspring response to live challenge

Data for 3.3.2. was collected only from the subset of chicks housed on wire, where it was possible to assign faeces to treatments. Faecal oocyst counts were repeated three times with different samples from the same pen to provide an average. Therefore the pen was the independent unit in these analyses as it was not possible to assign chicks to faeces. Pens were duplicated in each cohort giving 4 replicate pens per treatment, with 3 chicks per pen.

The mean oocyst output for each pen was analysed with a two way ANOVA blocked by cohort. This found no effect of substrate ($F_{1,11}$ =18.17, p = 0.249), treatment ($F_{1,11}$ =44.19, p = 0.084) or their interaction ($F_{1,11}$ =1.17, p = 0.763; Figure 3.3) on mean oocyst output (95% confidence interval for mean oocyst output; CoxAbic = 3.16-8.40, PBS = 6.40-11.63).

3.3.3. Mortality

Unfortunately when chicks died their leg rings and wing tags were quickly removed by the other chicks and so it was not possible to assign the few deaths to treatment groups. However, there were 4 deaths in cohort 1 (from 84) and 6 in cohort 2 (from 94).

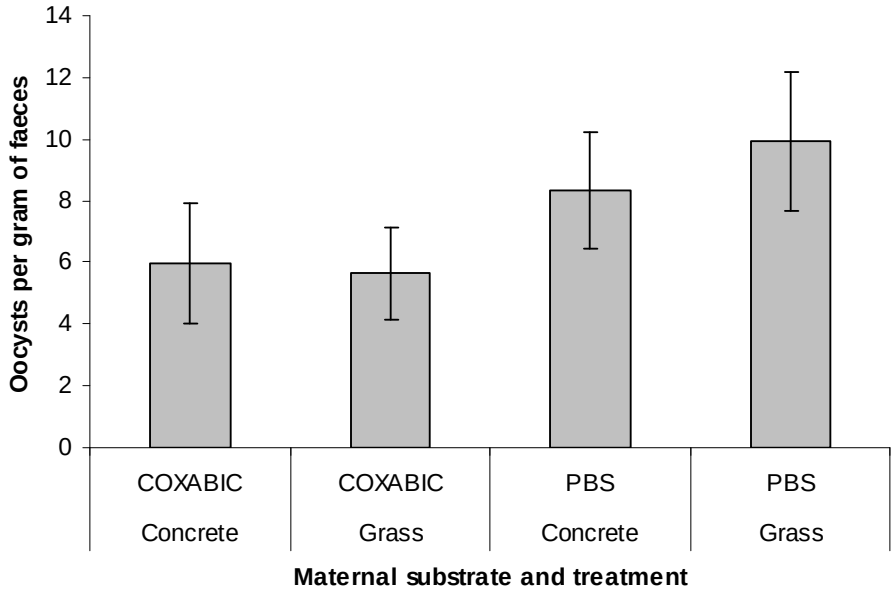

Figure 3.3: The effect of maternal treatment and maternal substrate on mean chick oocyst output (±se)

3.4 Discussion

Throughout the chick growth period maternal treatment had no detectable effect on chick weight and condition at hatching, pre and post challenge or final weight. However, chicks from mothers raised on grass and chicks hatched two weeks later were in better condition following challenge than earlier hatched chicks and chicks from mothers raised on concrete. There was also a significant effect of egg size and maternal condition at laying on chick condition up to three weeks of age.

Although there was no evidence for a significant effect of treatment on chick growth there was a non-significant trend in the expected direction for chicks from CoxAbic treated mothers to produce less oocysts. Despite there being no evidence for any effect of treatment on chick condition it does appear that it may have an effect on oocyst output. This is somewhat consistent with the action of a transmission blocking vaccine as the live challenge will still have lead to invasion of the gut epithelium and the associated damage, but appears to have somewhat reduced the number of released oocysts, potentially through the action on the wall forming body. It is possible that a larger challenge dose could have resulted in a stronger treatment effect on oocyst output. However, the oocyst dose was chosen based on the work of Liou *et al.* (2001) that suggested that oocyst output actually decreased with increasing dose and that even a twenty fold increase in oocyst dose had little effect (9g difference final weight between 100 and 2000 oocyst dose groups) on growth rate. Therefore our challenge dose was chosen to reduce the risk of mortality, and increase the number of oocysts excreted, therefore reducing suffering to the birds and providing us with a large range of excreted oocyst values. There are a number of

other potential explanations including differing virulence between the strains used in the experiments. However, it is apparent from overlapping confidence intervals that our study had insufficient power to fully explore the effect of treatment or substrate on oocyst output and future studies should use more birds, or re-examine the effect of oocyst dose on oocyst output under known conditions.

Hen substrate was a poor predictor of chick condition and weight throughout most of their growth period. However, it had a significant effect on final condition at week 7. These findings support those in chapter 2 that found that hens raised on grass were in better condition and invested more in total reproductive effort. More generally it has been found that parents in better condition produce chicks in better condition (Bolton, 1991; However see Arnold *et al*., 2006 where parental quality had only a small effect), and that the presence of high protein food sources, such as the insects available to hens raised on grass, can have positive effects on egg provisioning and subsequent offspring survival (Torok *et al.*, 2007). This could be due to a number of effects, for example hens in better condition could lay better provisioned eggs that enable the chicks to grow more quickly and invest in their own condition and immune system, thereby making them more resilient against infection. This may be seen in studies where hens in better condition laid heavier eggs (see Chapter 2) that produced heavier chicks and chicks of higher body condition up to three weeks of age (see 3.3.1). As well as these positive effects associated with hens raised on grass it is also possible that the females raised on concrete were more stressed (as discussed in Chapter 2), and that this may have led to them producing low quality

eggs and low quality offspring, such as has been previously seen in barn swallows (Saino *et al.*, 2005).

Hatching date was a good predictor of final chick weight and condition and unlike the hens it was later hatched chicks that were heavier, and in better condition. However, earlier hatched chicks were heavier up to three weeks of age, after which time catch-up growth in the later hatched chicks had compensated for their lower hatching weights. It is possible that weather may have also had an affect as later hatched chicks experienced much lower rainfall and more hours of sun than previous months. Rainfall has been shown to have a significant affect on chick growth in a number of species, but especially precocious species such as the Galliformes (Green, 1984; Moss, 1986; Beintema and Visser, 1989) where rainfall reduces the amount of time chicks can forage, as well as some evidence that it can directly affect thermoregulation (Tyler and Green, 2004; Greno *et al.*, 2008) in other avian species.

Previous work had shown that chicks tended to lose legs rings and so at hatching each chick was given an identical leg ring on each leg to allow for the loss of one ring and identically numbered patagial tags. At two weeks and four weeks the leg rings were swapped for larger sizes, again with the same number. However, even with these precautions in place many chicks still lost all their identifying tags. Other identification methods are available, for example tattooing, or Passive Integrated Transponder (PIT) tags. Both of these methods are more likely to remain with the chick, but both carry drawbacks as well such as the difficulty and ethics of tattooing

small chicks, and the price of PIT tags. However, future studies should explore these costs and benefits more fully.

This experiment still had significantly more individuals than the original studies by Wallach *et al.* which had 4-7 chicks per treatment group. We therefore expected to have significantly greater power to detect effects. However, this study found a number of non-significant trends, in the expected direction that were obscured by high variance, for example see Figure 3.3 which shows the potential benefit of the CoxAbic vaccine by reducing oocyst output. It is likely that with a larger sample size these effects may have been significant. One explanation for the lack of power, despite the larger sample size than previous studies could be due to the use of the vaccine in a different system, where it is not as effective at producing a protective immune response, potentially leading to a smaller effect size. Furthermore the pheasant system is much less sterile and controlled than the poultry system, potentially leading to greater variation between females due to different exposure histories as well as a less genetically homogenous population.

A further limitation of this study is the lack of an unchallenged control group of chicks. Without this group it is not possible to say whether the challenge was effective and therefore it's not possible to tell if the lack of an observed effect is truly due to there being no effect of treatment, or that challenge simply had no effect on the measured variables. A control group was not used in this experiment as there was insufficient space on the available concrete to run the minimum of two pens needed to house challenged and unchallenged chicks. Furthermore dividing the chicks like

this would have introduced a possible housing effect as a further confounding variable. This could be countered with a much larger study with multiple pens for challenged and unchallenged chicks, preferably with subdivided rearing pens such that challenged and unchallenged chicks could be reared within the same unit, but without coming into direct contact with each other. This would help to remove some of the variation that would likely occur between rearing houses.

Overall, this chapter shows that the CoxAbic vaccine had no measureable effect on chick growth or immunity and that it did not offer any significantly measureable protection against live challenge. However, this chapter has once again shown a significant effect of substrate and cohort. This confirms the findings of chapter 2 that substrate is important and that the effect of substrate on the hens can have long lasting effects on the offspring, especially when challenged.

Chapter 4 - Female immune responses to vaccination with the CoxAbic vaccine and live challenge with *Eimeria*.

4.1 Introduction

4.1.1. Immunity and life history trade-offs

It has been established in a number of systems that immune responsiveness can vary between individuals, as well as within individuals depending on a number of variables. Understanding the basis for this variation and the degree to which individuals vary in their response and how this relates to levels of protection conferred, is key to evaluating how effective any particular vaccine is likely to be in protecting a population from the costs associated with infection.

Some of the main effects on variation in immunity between individuals are caused by genetics (Råberg and Stjernman, 2003; Møller *et al.*, 2004) and their interaction with environmental effects (Christe *et al.*, 2000), such as the mother's condition during gestation (Kilpimaa *et al.*, 2007; but see Grindstaff et al., 2005), the offspring's rearing conditions (Råberg *et al.*, 2003), and any immune factors transferred from the mother (Grindstaff *et al.*, 2006; Reid *et al.*, 2006). Genetics can also have a significant effect on MHC (major histocompatability complex) diversity in individuals, which can potentially have a significant effect on the humoral immune response (Messaoudi *et al.*, 2002; Olsson *et al.*, 2005; Ottova *et al.*, 2007). Furthermore, the genetic effect on the immune system includes variation in all

components of the immune system. This can result in familial similarities in investment in different arms of the immune system, potentially resulting in misleading assumptions of lack of investment when only examining one aspect of immunity, when in fact the individuals may instead be investing more heavily in alternative components (for example, innate versus acquired immunity).

It has also been shown that body condition can have a significant effect on immune responsiveness in a number of avian systems. For example, there is some evidence that individuals in better condition are more able to produce a greater humoral immune response, measured as antibody response (Svensson *et al.*, 1998) and greater cell mediated immune response, as measured by PHA responsiveness (Lifjeld *et al.*, 2002). This potentially has important implications for juvenile development. For example, body condition is, in part, determined by energy availability, as well as genetic factors that determine the acquisition, utilisation and storage of energy. Therefore individuals in the same environment are likely to vary in their immune responsiveness depending on the aforementioned genetic factors.

One of the most important causes of within-individual variation is age. This can have potentially strong effects on immune responsiveness (Arlt and Hewison, 2004; Vleck *et al.*, 2007), both in terms of humoral (Saino *et al.*, 2003a) and cell mediated immunity (Lavoie *et al.*, 2007; Palacios *et al.*, 2007). This is generally termed immunosenescence and is a phenomenon that has been studied in a number of organisms and is linked to the general senescence of organisms. Exposure to stressors such as reproduction (Råberg *et al.*, 1998; French and Moore, 2008) and

other stressful activities such as restraint, exercise and cold can also adversely affect immunocompetence (Hoffmangoetz and Pedersen, 1994; Nieman and Nehlsen-Cannarella, 1994; DeVries *et al.*, 1997), potentially through the action of stress related hormones such as corticosterone (Besedovsky *et al.*, 1985; Besedovsky and DelRey, 1996; Ottaviani and Franceschi, 1996).

Other hormones, as well as the stress related glucocorticoids, can have significant effects on immunocompetence, for example prolactin (Yu-Lee, 2002) and leptin (Lord *et al.*, 1998) have both been shown to have immunosuppressive effects. Androgens (such as testosterone) and melatonin are two other important immunomodulatory hormones that can potentially play a large role in variation in immunocompetence. These are of particular importance because of their tendency to fluctuate throughout the year, for example with breeding season (testosterone) (Braude *et al.*, 1999; Roberts *et al.*, 2004; Muehlenbein and Bribiescas, 2005; Greives *et al.*, 2006; Hau, 2007) and day length (melatonin) (Nelson and Demas, 1996; Nelson and Drazen, 2000; Srinivasan *et al.*, 2008), though prolactin also fluctuates seasonally (Goldman and Nelson, 1993). Androgens appear to have a suppressive effect on the immune system (Braude *et al.*, 1999; Roberts *et al.*, 2004; Greives *et al.*, 2006). However, it is possible that although androgens may directly suppress the immune system, the potential increase in dominance and associated greater access to resources may offset this cost (Evans *et al.*, 2000). On the other hand melatonin generally enhances the immune response (Nelson and Demas, 1997; Hotchkiss and Nelson, 2002; Carrillo-Vico *et al.*, 2006; Srinivasan *et al.*, 2008), but

has been shown to reduce the cell mediated immune response in hamsters (Prendergast *et al.*, 2001)

4.1.2. The avian immune system

In order to better understand the mechanisms behind the possible costs and benefits of immunity it is necessary to understand the basics of the avian immune system. The avian immune system is a highly complex set of cellular mechanisms that help to control, prevent and eradicate infection. The immune system is comprised of two main components, the innate and the acquired immune systems. The innate immune system is usually the first to be triggered, and provides a very non-specific response against any non-self object (Muller *et al.*, 2008), such as helminths, bacteria or foreign bodies (for example splinters or debris trapped in wounds). The acquired immune system adapts itself in response to infections and is responsible for cell mediated immunity and the humoral immune response and provides a specific and targeted response, as well as forming the basis of immunity through immunological memory (Gershon *et al.*, 1971).

A simple outline of a typical immune response can be seen in Figure 4.1. The innate immune system is important in phagocytosing foreign bodies, cellular debris, and digesting pathogen derived molecules (antigens) via macrophages and phagocytes (1). Digested antigens are displayed on antigen presenting cells (APCs) which are important in triggering helper T-cells, part of the acquired immune system. Once active helper T-cells undergo replication and secrete cytokines which in turn activate the T-cell cascade (2). Killer (Cytotoxic) T-cells, a significant part of cell mediated

immunity, replicate (3) and bind to the antigens that were presented to the helper T-cells, causing cell lysis of any cell displaying the antigens. The resulting chemicals attract other immune cells such as macrophages which phagocytose the cellular debris. During the replication process some cytotoxic T-cell daughter cells mature in to memory T-cells which are important for immunological memory. Activation of the T-cell response leads to activation of the B-cell response, and vice versa via a positive feedback loop. B-cells activate when they bind to an APC bound antigen in the presence of T-helper cell cytokines (4). They then undergo clonal selection eventually leading to production of antibodies (5). Antibodies can bind to the surface of the pathogen and trigger the complement system, killer T-cells, natural killer cells and macrophages to lyse, perforate and engulf the foreign cells. Once the infection has passed the activated B-cells become memory cells which can rapidly respond and stimulate antibody production if the antigen is encountered again (6).

Antibodies are a class of immunoglobulins (Ig) and, unlike mammals, birds posses IgY (Lundqvist *et al.*, 2006), which is functionally similar to IgG and is thought to be an evolutionary precursor to both IgG and IgE. IgG in mammals (Parvari *et al.*, 1988; Magor *et al.*, 1994; Warr *et al.*, 1995), and IgY in birds plays a major role in the control of extracellular pathogens (Warr *et al.*, 1995). However, during an *Eimeria* infection IgA is produced, which is the main mucosal antibody. This is present in small quantities in the blood which is how injection of *Eimeria* antigens is able to induce an immune response in the intestine, where levels of IgG/Y are low. However, the assay used to detect *Eimeria* infection is based on measuring levels of IgG/Y, present at much higher quantities in the blood.

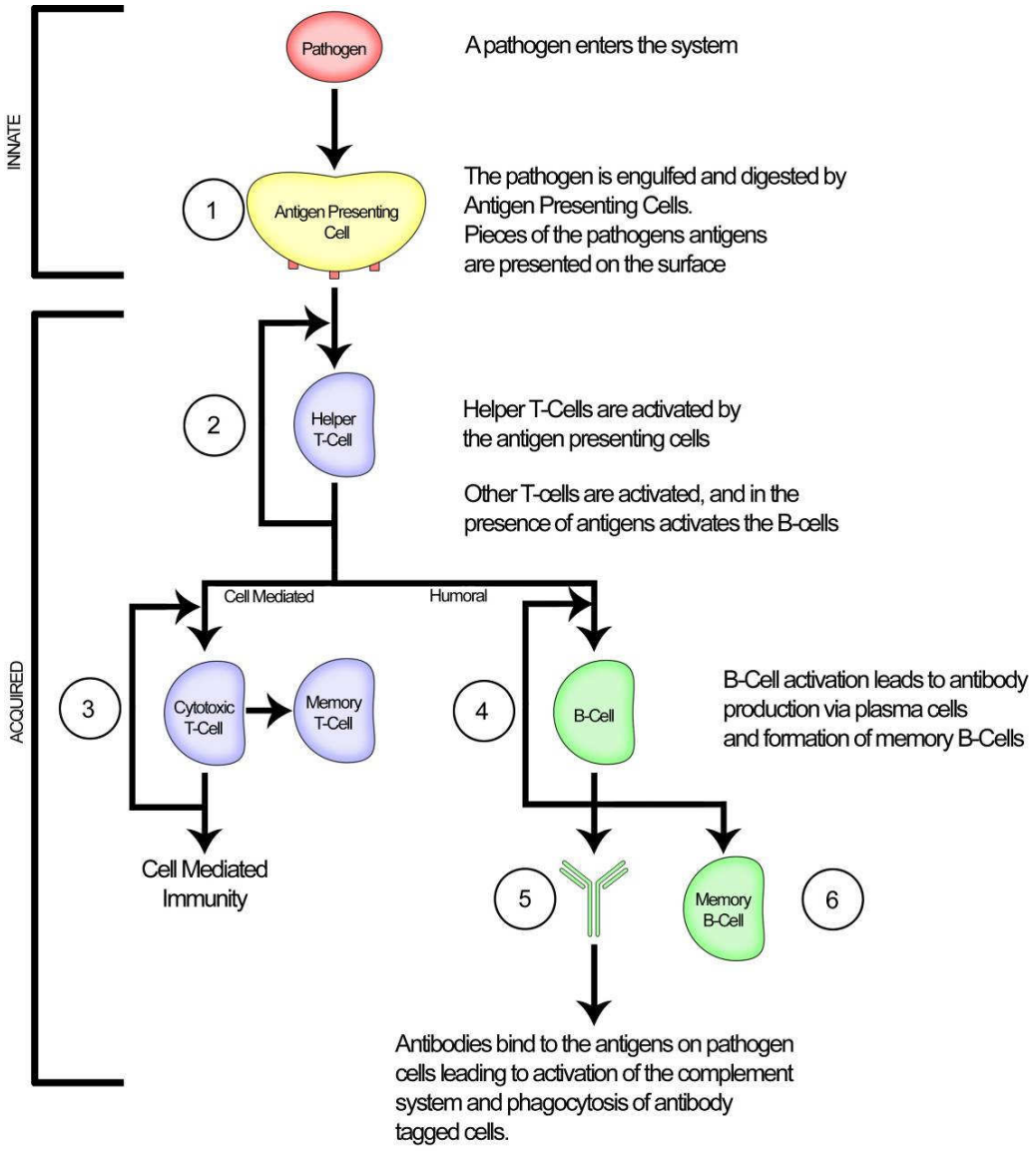

Figure 4.1: A simplistic overview of the avian immune system showing a generalised immune response to a pathogen such as *Eimeria*. See main text for further details.

4.1.3. Immunology of *Eimeria* infections

Although coccidiosis is very common the mechanism of the interactions between *Eimeria* and the host immune system are not fully understood, but they are reviewed well by Lillehoj *et al.* (1996). The main protective response to *Eimeria* infection appears to be due to cell mediated immunity, specifically T-cell related responses (Lillehoj and Trout, 1993; Lillehoj and Trout, 1994; Trout and Lillehoj, 1996; Lillehoj, 1998). It has also been found that bursectomised birds that are therefore unable to produce antibodies, are still capable of developing immunity against infection (Giambrone *et al.*, 1981; Lillehoj, 1987) confirming strong involvement of the other arms of the immune system. However, antibodies can also offer protection (Girard *et al.*, 1997) against sporozoite invasion (Rose *et al.*, 1984; Rose and Hesketh, 1987; Guzman *et al.*, 2003; Constantinoiu *et al.*, 2008) and help in the phagocytosis of merozoites (feeding stages) (Onaga and Ishii, 1980; Bekhti and Pery, 1989) with IgY being the most prolific immunoglobulin produced in response to oral vaccines (Ayaz *et al.*, 2008), although some studies only found inconclusive or weak effects of antibodies on *Eimeria* infections (Speer *et al.*, 1985; Augustine and Danforth, 1986). Furthermore, antibodies have been shown to be important for the maternal transfer of protective immunity to offspring against coccidiosis (Rose, 1972; Rose *et al.*, 1988; Wallach *et al.*, 1990; Smith *et al.*, 1994b) and other diseases (Ahmed and Akhter, 2003; Al-Natour *et al.*, 2004; Decaro *et al.*, 2005; Grindstaff, 2008; Hasselquist and Nilsson, 2009) through their ability to be transferred to young.

This chapter is based on the experiments conducted in chapter 2 and will look at the effectiveness of the CoxAbic vaccine at raising an immune response in hens by

looking at IgY titres. These responses will be compared to those of hens treated with the adjuvant or PBS and those gained from environmental exposure alone. It will also look at how raising an immune response affects reproductive investment and investment in other life history traits such as maintenance. These will be assayed using direct measures or counts of the trait in question. For example, maintenance will be looked at by measuring weight and body condition index, and reproductive investment will be examined by counting the number of eggs laid and measuring egg volume, egg weight and total reproductive effort. Each of these will be looked at in relation to the individual immune responses, measured as circulating IgY titres, assayed through serum OD values.

4.2. The effect of CoxAbic and the associated adjuvant on immune responses

4.2.1. Methods

To recap, twenty-seven female and three male birds were weighed (to the nearest 20 grams) and morphometrics taken (tarsus to the nearest mm). A baseline blood sample was taken and from this point forward weekly blood samples were taken from all females to give a complete time series for the IgY titres. All blood samples were taken from the vena brachialis by puncturing the vein and drawing 150 μl of blood into a heparinised capillary tube and then deposited into a 1.5ml eppendorph. The serum was isolated by centrifuging the samples for 5 minutes at 900 rpm. The supernatant fraction of serum was then poured off into a fresh 1.5ml eppendorph, labelled and stored at -20^{o}C until used for analysis.

Females were then randomly divided into three treatment groups; PBS (n = 9), FIA (Freund's incomplete adjuvant; n = 9) and CoxAbic (n = 9) (Figure 4.2). A one-way ANOVA was performed using baseline body condition index (BCI) as the dependant variable to make sure that no difference existed between the groups by chance ($F_{2,24}$ = 0.01, p = 0.913). All birds were housed on concrete in 10' x 40' pens with pen furniture as previously described. Food was provided *ad libitum* by two 20 L hoppers per pen, and water by 3 overhead nipple drinkers (Quill Productions Ltd) per pen. All birds were fed Pheasant Breeder Pellets (Gamekeepa Feeds) consisting 28% protein, this feed company specialises in organic game feeds and so all feeds used throughout were free of all chemical prophylaxes, including coccidiostats. Multiple (three) mating pens were used with one male per pen as pheasants are naturally harem breeders with a male holding a harem of up to 12 females. Hens of different treatments were mixed evenly between pens to ensure there was no confounding housing or mate effect.

Hens were allowed to acclimatise for one week, and then on the day of treatment 0.5 mL of each treatment was administered to all hens. All treatments were given blind. Four weeks later the treatment regime was repeated with all hens receiving the same dose of the same treatment. After one week the females were housed individually and allowed to lay on concrete in 5' x 10' pens. Eggs were collected twice a day from 1100h - 1200h and from 1700h-1800h. All eggs were cleaned, weighed and measured (width and breadth) before being stored. After ten days all females were returned to their communal pens

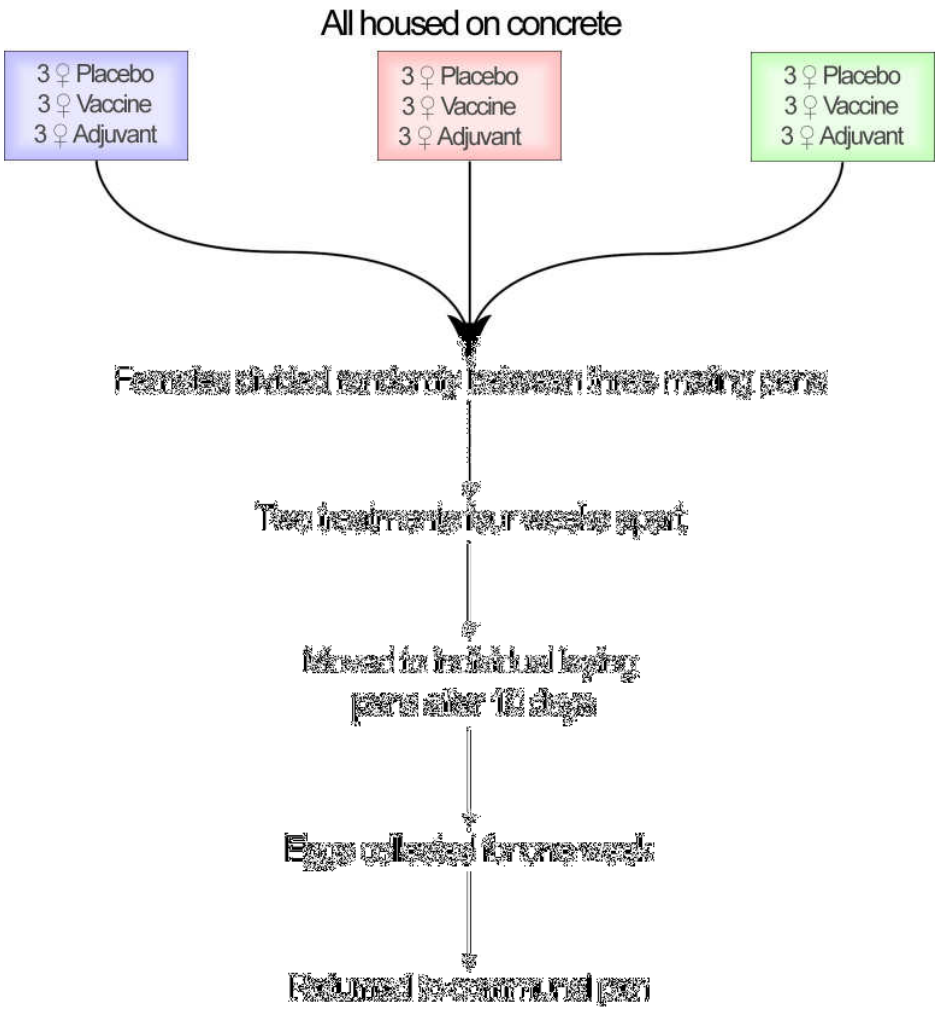

Figure 4.2: Schematic of methods for 2006.

4.2.1.a. ELISA technique

Levels of IgY produced in response to the different treatments were measured using the CoxAbic proprietary enzyme linked immunosorbent assay (ELISA) kit (Abic Biological Laboratories Teva, Israel). This kit came with 96-well plates pre-coated with the *Eimeria maxima* gametocyte antigens used in the vaccine (Figure 4.3; 1), and all solutions pre-made, but requiring dilution. The PBS buffer was prepared by diluting PBS and BSA x 10 in a 1:10 ratio in distilled water. Sera were diluted 1: 50 in this buffer and 100 μL of each serum was transferred into duplicate wells (two per sample), as well as two wells each for a high positive control serum, medium positive control serum, negative control serum and blank wells containing only PBS and BSA dilution buffer. Samples were allocated randomly across plates with respect to treatment and substrate. Plates were then covered and incubated at 37 ^{o}C for 90 minutes to allow the antibodies present in the serum to bind with the *Eimeria* antigens coated on to the walls of the plates (2).

All plates were washed three times with PBS-Tween to remove any unbound antibodies (3). Conjugate (Rabbit anti-chicken IgG-alkaline phosphatase) was then diluted 1: 1000 in the PBS and BSA buffer and 100 μL added to each well. Plates were covered and incubated at 37 ^{o}C for 60 minutes. During this step anti-chicken antibodies bound to the pheasant antibodies already bound to the *Eimeria* antigens. The rabbit anti-chicken antibodies were conjugated with an alkaline phosphatase enzyme that was important in the next step (4).

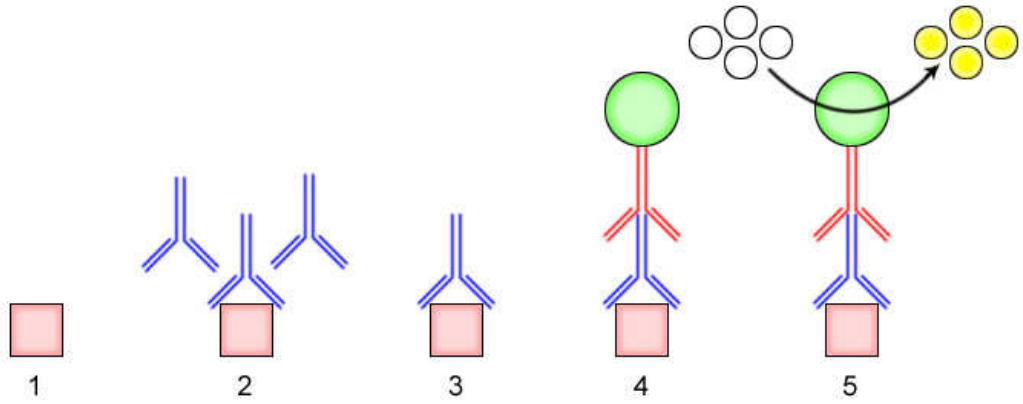

Figure 4.3: Overview of an ELISA. 1 – the plate is coated with *Eimeria maxima* gametocyte antigens. 2 – pheasant serum containing anti-*Eimeria maxima* antibodies is added. 3 – the excess antibodies are washed away leaving only antibodies that are bound to the antigens. 4 – rabbit anti-chicken antibodies bind to the pheasant anti-*Eimeria* antibodies. 5 – the enzyme attached to the rabbit anti-chicken antibodies converts ρ – nitrophenyl phosphate to ρ – nitrophenyl, turning the solution yellow.

The substrate was made up by dissolving two ρ – nitrophenyl phosphate tablets per plate in 10 ml of diethanolamine buffer (pH 9.8), and 100 µl was added to each well. Plates were protected from light, and after 5 minutes the optical density (OD) values were read at 405 nm. Optical density is a measure of the transmittance of light through a substrate at a given wavelength, such that the higher the optical density the more light the solution absorbs. The plates were read repeatedly until the high positive serum reached an OD_{405} of 1.3. This optical density was chosen by Abic as it represents a high level of response to the positive serum within an accurately readable range by the machines, and allows for a range of detectable values to account for individual variation below this. In the final step the antibody linked enzyme digested the substrate to ρ – nitrophenol, a coloured solution with an absorbance at 405 nm (5). By measuring the optical density at 405 nm it was possible to assess the amount of ρ – nitrophenol formed, and therefore the amount of pheasant IgY antibody present. The amount of IgY present in the sera was analysed using the average OD_{405} of the serum duplicates, minus the OD_{405} of the buffer only wells. This corrected OD_{405} measurement is hereafter referred to as the OD values.

4.2.2. Statistical analyses

Repeated measures linear mixed models were used to examine the effect of treatment on serum OD values from baseline (week 1) to one week after the vaccination regime (week 7) in the 2006 season and of the effect of treatment on egg characters. Week 7 was used because it was one week after the end of the vaccination regime, and therefore titres should have been at their highest (Ziomko *et al.*, 2005). The assumptions of sphericity were not met and so the model degrees of freedom were

corrected with the Greenhouse-Geisser epsilon value (0.54). Models exploring the effect of treatment on egg weight and volume included serum OD value at week 5 (the week eggs were collected) and treatment as fixed effects and female as a random effect to account for repeated eggs. Models exploring the effect of treatment on total reproductive effort and clutch size did not include female as a random effect as there were no repeated measures.

4.2.3. Results

4.2.3.a. What effect do the different components of the CoxAbic vaccine have on the immune system?

There was no evidence for any effect of treatment on serum OD values from baseline to one week after the end of the vaccination regime ($\chi^2_{1.08,\ 100.98} = 0.14$, $p = 0.709$; Figure 4.4a). This effect was also looked at by examining the change in serum OD values from week 1 to week 7. This found no significant effect of treatment on the change in serum OD values ($\chi^2_{25} = 5.71$, $p = 0.057$, Figure 4.4b).

4.2.3.b. What effect does an immune response have on egg characters?

There was no evidence for any effect of treatment ($\chi^2_{2,\ 323} = 2.24$, $p = 0.107$), or serum OD values ($\chi^2_{1,\ 322} = 0.77$, $p = 0.381$) on egg weight or volume (treatment ($\chi^2_{2,\ 323} = 2.93$, $p = 0.231$), serum OD values ($\chi^2_{1,\ 322} = 1.16$, $p = 0.281$)). There was also no effect of treatment ($\chi^2_{2,\ 23} = 1.76$, $p = 0.415$) or serum OD values ($\chi^2_{1,\ 22} = 0.10$, $p = 0.749$) on clutch size or total reproductive effort (treatment ($\chi^2_{2,\ 23} = 3.28$, $p = 0.194$), serum OD values ($\chi^2_{1,\ 22} = < 0.001$, $p = 0.988$)).

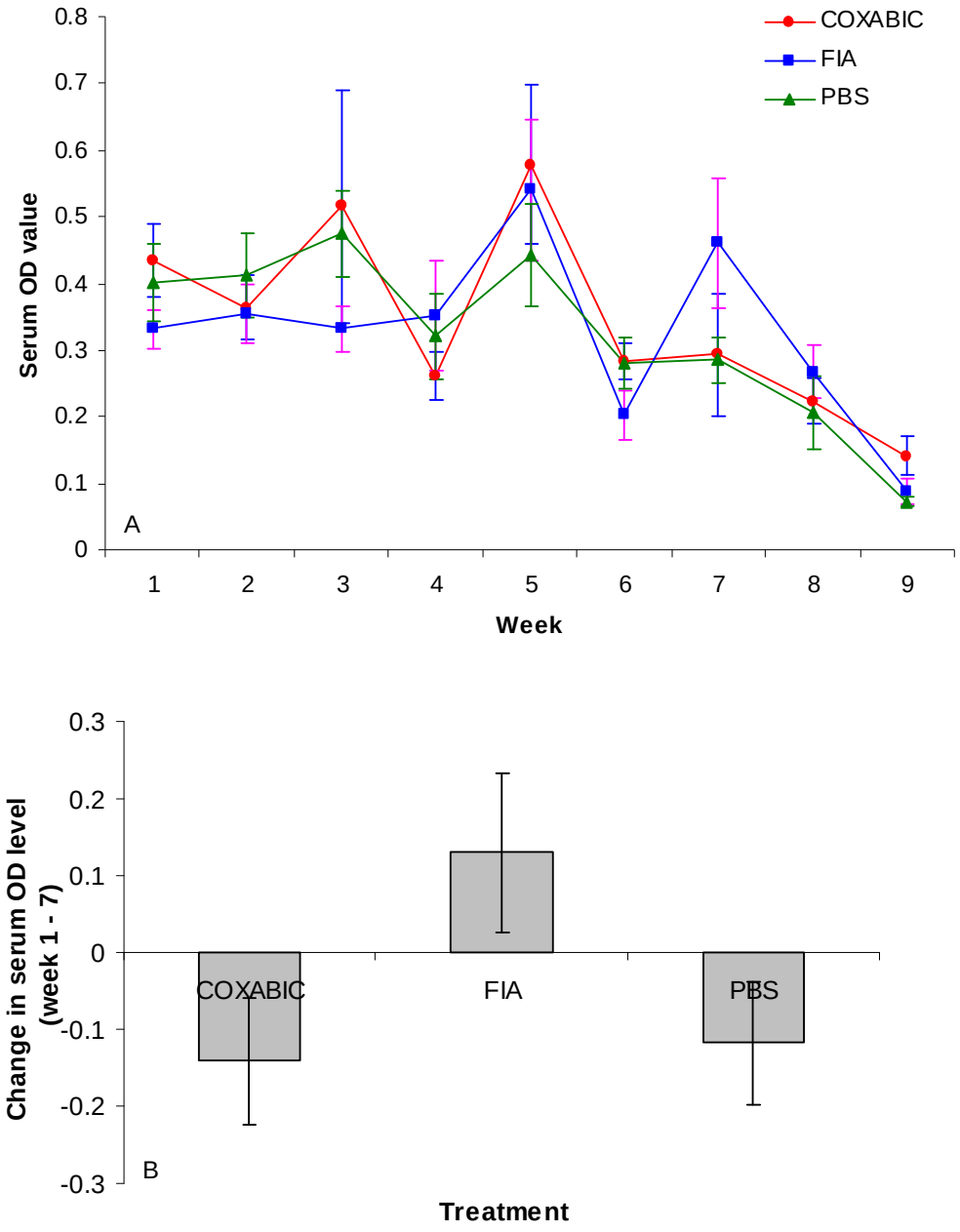

Figure 4.4: A. The effect of treatment on mean serum OD values throughout 2006 (±se) from baseline to one week after the end of the vaccination regime. B. The change in serum OD levels from week 1 to week 7 (±se)

4.3. The effect of CoxAbic and rearing substrate on immune responses

4.3.1. Methods

Because no difference was detected in 2006 between the treatment groups it was decided to remove the FIA treatment to give more power to focus on the effects of the vaccine relative to the placebo. It was also noted that throughout 2006 the hens, who were all housed on concrete, showed a slow loss of IgY titres regardless of treatment. This was most likely due to the lack of environmental exposure as the concrete was regularly cleaned. Therefore in 2007 it was decided to house half of the birds on grass and half on concrete so that the effect of environmental exposure in the absence and presence of the vaccine could be examined to see if the vaccine offered any benefits above and beyond that offered by environmental exposure alone.

The methods for 2007 were very similar to 2006 but with some important differences (Figure 4.5). Eighty hens were used, randomly divided between two cohorts spaced two weeks apart. Half of each cohort was housed on grass and half on concrete. Birds were housed on either grass or concrete to allow comparison between normal housing conditions (grass), where birds inevitably encounter low levels of a wide range of environmental pathogens (including coccidiosis), and a clean environment (concrete) where there should have been little environmental exposure. Only hens with a complete set of blood samples were analysed.

4.3.2. Statistical analyses

Throughout analysis the hen was treated as the independent unit. The results were analysed using residual maximum likelihood (REML) analysis with treatment as a fixed effect. Each model examined the effects of substrate and treatment alone to check for their effects before exploring the model from the top down. The models used are discussed in more detail at their point of use. Data were tested for sphericity using the Mauchly criterion. Where the assumptions of sphericity were not met the model degrees of freedom were corrected using the calculated Greenhouse-Geisser epsilon values and the *p* value recalculated.

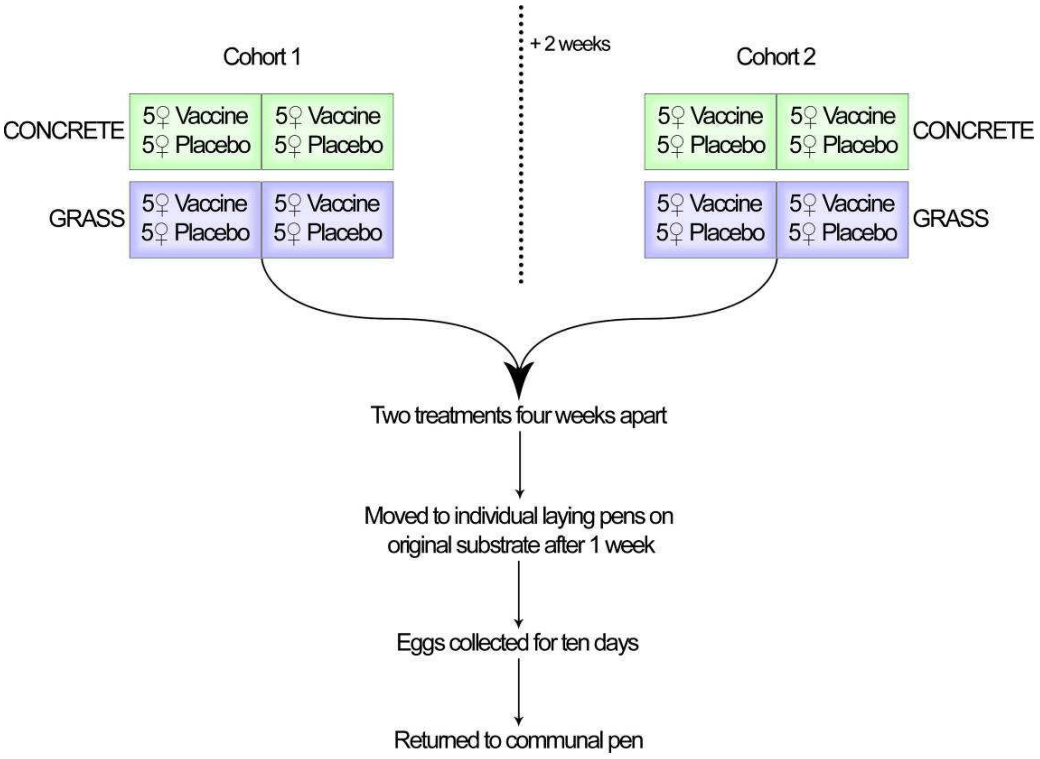

Figure 4.5: Schematic of the methods for 2007

4.3.3. Results

4.3.3.a. How does vaccination affect the change in antibody titres in the presence and absence of environmental exposure?

This was examined with data from 2007, which only had serum OD time points for weeks 1, 7 and 14 from the start of the experiment. This question was analysed using a linear mixed model with change in serum OD values from week 1 to 7 as the dependant variable and substrate, treatment (and their interaction) and cohort as fixed effects. There was no evidence for a significant independent effect of substrate ($\chi^2_{1, 63}$ = 2.87, p = 0.090; Figure 4.6A), treatment ($\chi^2_{1, 63}$ = 1.17, p = 0.279) or cohort ($\chi^2_{1, 62}$ = 0.21, p = 0.646) on the change in serum OD values. However, there was a significant effect of the substrate by treatment interaction ($\chi^2_{1, 63}$ = 8.12, p = 0.004). This shows that the effect of treatment depended on the rearing substrate such that hens housed on grass and treated with PBS had the highest OD values, whereas hens on concrete treated with PBS had the lowest values.

Lastly the changes in serum OD values from week 1 to week 14 were looked at with the same model. There was no evidence for any effect of treatment ($\chi^2_{1, 64}$ = 0.32, p = 0.571), substrate ($\chi^2_{1, 62}$ = 0.09, p = 0.760), their interaction ($\chi^2_{1, 62}$ = 0.36, p = 0.549; Figure 4.6B) or cohort ($\chi^2_{1, 64}$ = 0.48, p = 0.487).

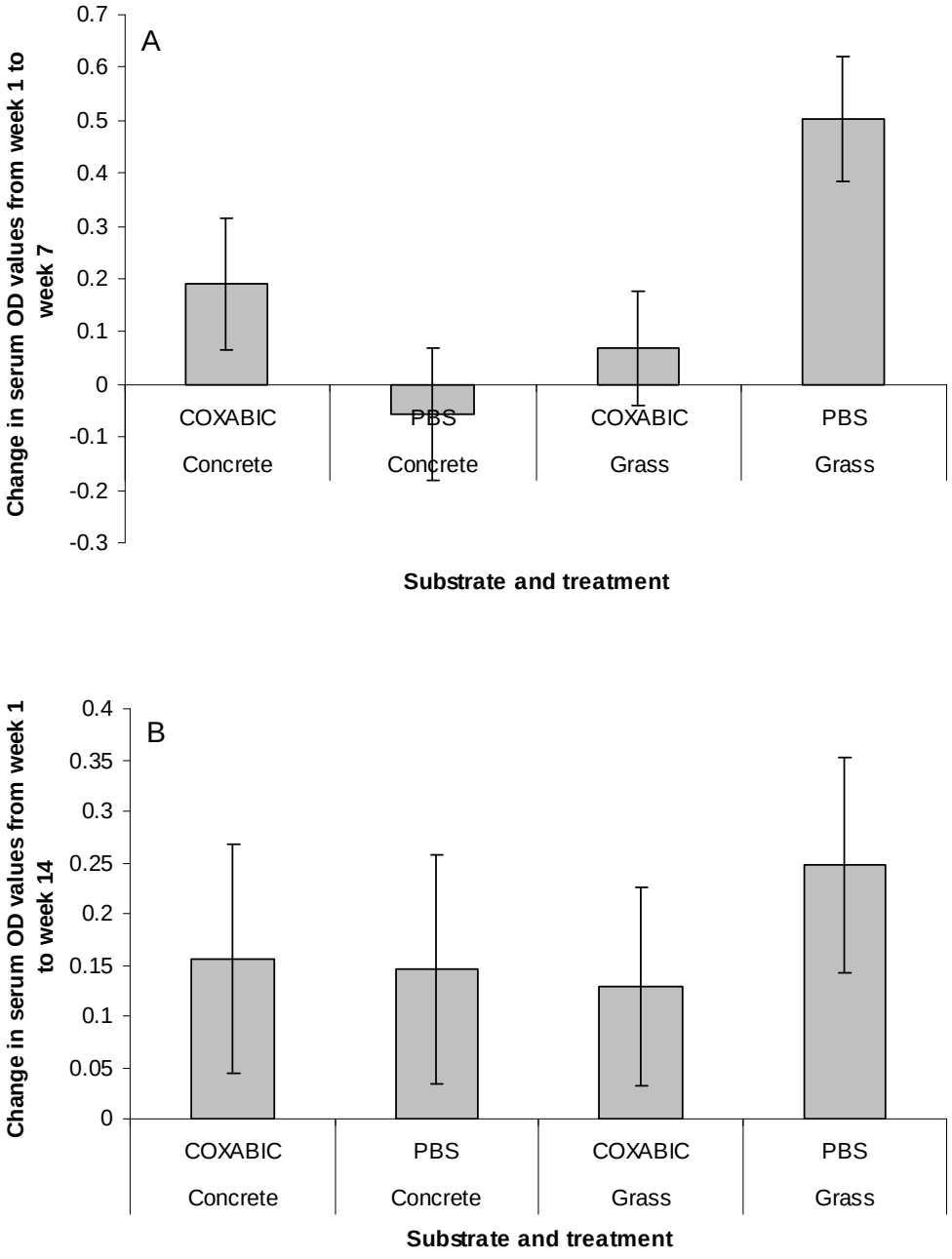

Figure 4.6: The effect of treatment and substrate on the mean change in serum OD values (±se) from baseline to one week after the vaccination regime (A) and from baseline to week 14 (B) for birds from 2007. Means shown are estimated from the model.

4.3.4. Factors associated with individual variation in immune responses

The antibody titre data analysed in sections 4.3.1 and 4.3.2 was also examined in conjunction with other life history characteristics to look for potential trade offs between immune function and other costly traits. The two life history traits that were perhaps most likely to have been affected by raising an immune response were body condition and total reproductive effort. The data allowed examination of the correlation between body condition and serum OD values before and after vaccination, and the correlation between total reproductive effort and serum OD values. The change in serum OD values from week one to week seven were first examined with a one way ANOVA blocked by individual to see if there was a significant change through time. This found that there was a significant difference in the mean serum OD values at week one and week seven ($F_{1,93} = 5.57$, $p = 0.002$) and so more detailed analyses were conducted.

4.3.4.a. How do Serum OD values correlate with body condition?

The effect of body condition index on baseline OD values was examined with a linear mixed model including data from 2006 and 2007. Substrate, treatment and cohort were not included in this model as birds had not yet been treated, housed differently or separated into cohorts before this point. There was no evidence for a significant effect of baseline serum OD values ($\chi^2_{1, 90} = 0.33$, $p = 0.566$) or year ($\chi^2_{1, 90} = 3.03$, $p = 0.082$) on baseline body condition index. However, there was a significant negative effect of the year by baseline OD value interaction ($\chi^2_{1, 90} = 6.94$, $p = 0.008$; Figure 4.7) such that the negative relationship between body condition

index and baseline serum OD values was more pronounced in 2006. This result is similar to that found in Figure 2.6, but includes data from birds in 2006.

The correlation between the change in serum OD values and body condition index from baseline to one week after the end of the vaccination regime was examined next. Only data from 2007 was used as there was no weekly weight data available for hens from 2006. There was no evidence for a significant effect of treatment ($\chi^2_{1, 62}$ = 0.39, p = 0.530), substrate ($\chi^2_{1, 62}$ = 1.34, p = 0.247), the substrate by treatment interaction ($\chi^2_{1, 60}$ = 0.04, p = 0.837), cohort ($\chi^2_{1, 61}$ = 0.06, p = 0.805), or the change in serum OD values ($\chi^2_{1, 60}$ = < 0.01, p = 0.955), on the change in body condition index

Finally, the correlation between the serum OD and the final body condition index for hens in 2007 was examined. The final time point was one week after the hens were given a live challenge of oocysts. Therefore the presence of any correlation between body condition and the ability to mount a response to live challenge could be explored. The final body condition index was the dependant variable with substrate, treatment (and their interaction), final serum OD values and cohort as fixed effects. Substrate was found to have a significant effect on hen condition ($\chi^2_{1, 64}$ = 7.81, p = 0.005), with hens on grass being in better condition, confirming the findings of 2.3.3.c. However, there was no evidence for any effect of treatment ($\chi^2_{1, 61}$ = 0.55, p = 0.457), the substrate by treatment interaction ($\chi^2_{1, 61}$ = 1.44, p = 0.230), cohort ($\chi^2_{1, 61}$ = < 0.01, p = 0.950) or the final serum OD values ($\chi^2_{1, 64}$ = 2.52, p = 0.113) on final body condition.

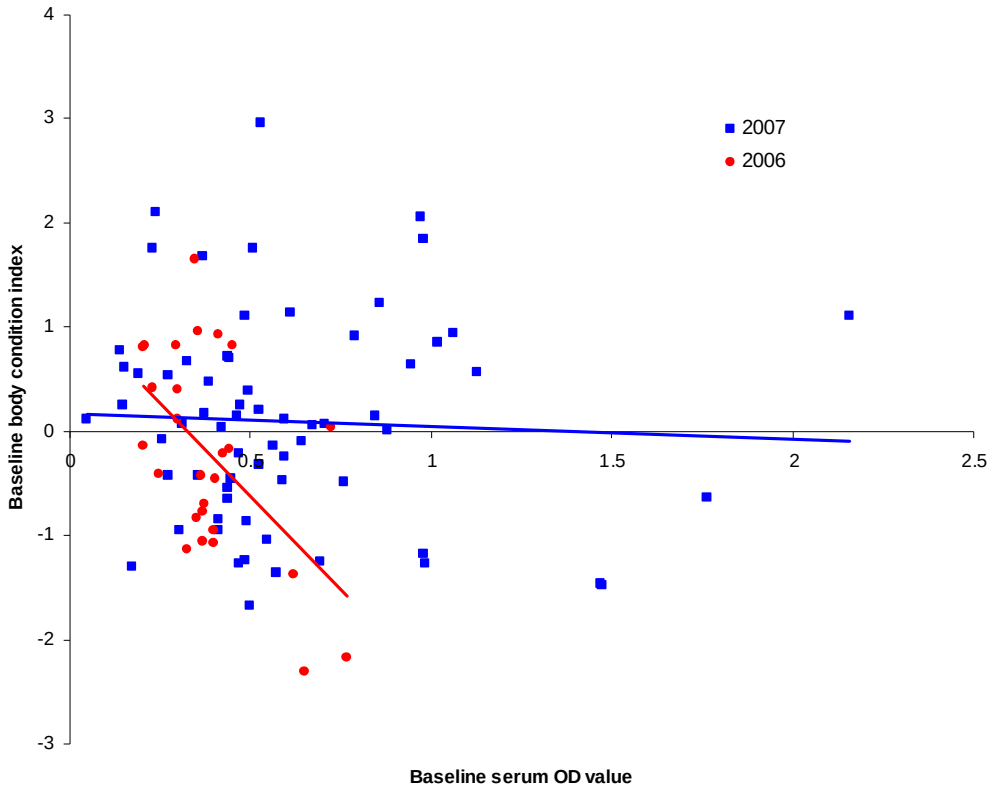

Figure 4.7: The correlation between body condition index and baseline serum OD values for hens treated with CoxAbic or PBS with trend lines.

4.3.4.b. How do serum OD values correlate with reproductive characteristics?

The effect of serum OD values at week 7 (one week after vaccination and one week before egg laying) on reproductive characteristics (egg volume, egg weight, clutch size and total reproductive effort) in 2006 and 2007 was examined in hens laying on concrete alone as this was the only substrate group in 2006. These models were similar to those in 2.2.3.a, but included serum OD values as an additional fixed effect along with treatment, cohort and year. Individual was included as a random effect, but individuals recorded as non-layers were excluded from these analyses as it was not possible to tell if they were not laying, or were eating their eggs.

The effect of serum OD values on egg volume was examined first. Hens in 2006 were found to lay heavier eggs ($\chi^2_{1,\ 452} = 4.42$, $p = 0.036$). However, there was no effect of treatment ($\chi^2_{1,\ 453} = 2.27$, $p = 0.321$), cohort ($\chi^2_{1,\ 450} = 0.45$, $p = 0.501$), or serum OD values ($\chi^2_{1,\ 452} = 2.66$, $p = 0.103$) on egg volume.

The effect of serum OD values at week 7 on egg weight was examined next. There was no evidence for any significant effect of treatment ($\chi^2_{1,\ 451} = 2.76$, $p = 0.251$), serum OD value ($\chi^2_{1,\ 452} = 2.58$, $p = 0.109$), year ($\chi^2_{1,\ 452} = 3.40$, $p = 0.065$) or cohort ($\chi^2_{1,\ 450} = 0.71$, $p = 0.401$) on egg weight.

Clutch size was considered next. Hens in 2006 produced significantly larger clutches ($\chi^2_{1,\ 56} = 18.55$, $p = < 0.001$). However, there was no evidence for any effect of treatment ($\chi^2_{1,\ 53} = 0.22$, $p = 0.636$), cohort ($\chi^2_{1,\ 55} = 1.65$, $p = 0.199$) or serum OD values ($\chi^2_{1,\ 54} = 1.71$, $p = 0.191$) on clutch size.

As previously described, total reproductive effort is another way of examining the trade-off between egg size and clutch size. It is also another test on previously tested data which was accounted for with a Bonferroni correction by dividing the alpha value by the number of tests (2) to give a corrected alpha value of $p = 0.025$. Hens from 2006 invested more in total reproductive effort ($\chi^2_{1,56} = 20.10$, $p = < 0.001$). However, treatment ($\chi^2_{1,\ 53} = 0.48$, $p = 0.489$), cohort ($\chi^2_{1,\ 55} = 1.28$, $p = 0.259$) and serum OD values ($\chi^2_{1,54} = 2.52$, $p = 0.113$) had no effect.

Many more samples were collected in 2007 in order to look at the effect of maternal serum OD values on chick OD values, and to assess the effect of chick OD values on their growth and condition. However, it was noted during analysis of the 2007 samples that some of the 2007 samples contained mould which could potentially have interfered with the OD values, for example by increasing the optical density through deposition of particulates in the serum. Figure 4.8 clearly shows the difference in means between hens housed on concrete in 2006 and 2007. The samples to be analysed were chosen *a priori* based on time points of interest, for example, baseline, one week after vaccination (when the IgY response should have been highest) and at the end of the experiment. When it was found that the mould could have been affecting the OD values no further samples were analysed.

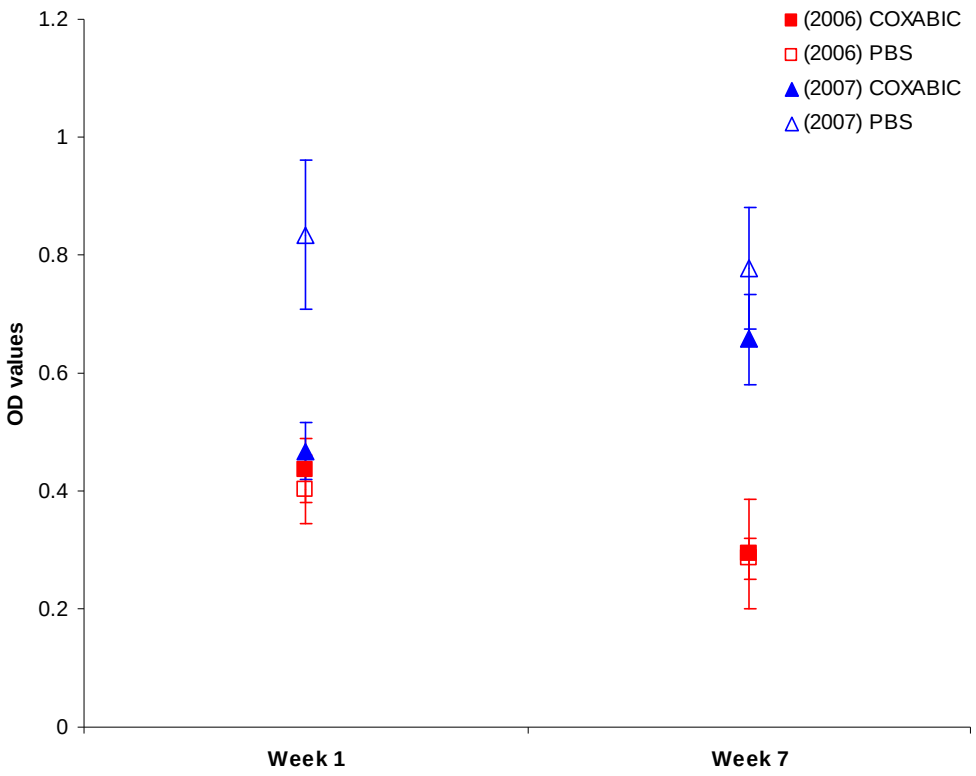

Figure 4.8: The mean serum OD values (±se) for hens on concrete during weeks 1 and 7 for 2006 and 2007.

4.4 Discussion

Taken as a whole these results show that neither treatment nor adjuvant had an effect on the change in serum OD values over and above environmental exposure and that the CoxAbic vaccine specifically had no effect greater than that of PBS. However, as with previous results there was often a significant (or near significant) effect of substrate on serum OD values and reproductive effort with birds on grass tending to produce higher titres, and invest more in reproductive effort. This suggests either that being housed on grass allowed birds to invest to a greater extent in reproduction and immunity or that low level exposure to coccidia in the birds on grass led to the higher titres. There was also a near significant effect of FIA on the change in serum OD values from week 1 to week 7 in 2006. However, this effect is inconsistent with the complete time series data which shows no effect of treatment across the entire experiment. Therefore this result should be treated with caution.

The different components of the CoxAbic vaccine do not appear to have had any measurable effect on the serum OD values of the hens. This shows that the effect of CoxAbic was no greater than that of Freund's adjuvant, and that neither of these components produced a measurable immune response relative to the placebo (PBS) treated birds. This appears to be in contrast to the work of Wallach *et al.* during the development of this vaccine which found strong effects of the coccidial antigens (Pugatsch *et al.*, 1989; Wallach *et al.*, 1995b) and Freund's complete adjuvant alone (Smith *et al.*, 1994a). Although it may be possible that this is due to the vaccine being developed in chickens this seems unlikely given the unpublished trials showing a protective effect of CoxAbic in pigeons. Post-hoc power analysis of most of the

models in this chapter showed a power of 20%-30% with 300-350 pheasants needed to detect a significant effect with the effect size and standard error observed in these experiments. This suggests that sample size may have been a limiting factor in these experiments and that future work should include at least 350 pheasants. However, as discussed in previous chapters, this is a much larger sample size than other experiments have used and found a significant effect.

The adjuvant used in preliminary work on this vaccine was Freund's complete adjuvant, which contains desiccated *Mycobacterium*, a strong immunogen. However, all subsequent trials and vaccine formulations contain Freund's incomplete adjuvant. This is essentially identical, except it does not contain desiccated *Mycobacterium.* This could potentially reduce, or negate the adjuvant effect as the incomplete adjuvant contains mineral oil which is used to slowly release the antigens into the body over time, but no *Mycobacterium*. The reasons for using the incomplete adjuvant are not discussed in any of the related literature. However, it is possible that it was used because of the possible side effects of the complete adjuvant which include localised necrosis and extreme pain. In order for our results to be comparable and relevant to its potential use in industry it was necessary to use the incomplete adjuvant, as is used in the vaccine preparation. However, the incomplete adjuvant may not have been immunogenic enough to stimulate a significant effect on its own.

There is some evidence that Freund's adjuvant can also alter the humoral immune response (Chuang *et al.*, 1997). In the context of this study this could potentially have amplified the response to naturally occurring coccidia that was likely to infect

birds housed on grass, potentially providing a more specific response than that from the vaccine subunits, which are based on chicken *Eimeria*. For example, pheasants housed on grass and therefore potentially exposed to *Eimeria* could, through the effect of the adjuvant, produce a more specific immune response than the pheasants housed on concrete and exposed to the chicken *Eimeria* derived antigens in the vaccine. However, this effect was not seen.

The lack of a response to the CoxAbic vaccine and Freund's adjuvant seen here could also be due to differences in the rearing practices between the poultry and game bird industries. The game bird rearing industry is a relatively "dirty" industry as the birds are often reared outside on grass, whereas the poultry industry rears birds inside on regularly disinfected and changed litter. This could potentially lead to a situation where game birds continually produce a sustainable response to coccidiosis, a pathogen which they regularly encounter, and that any extra antigenic stimulus is ignored as they are already producing their optimally sustainable titres. There is no data available on the serum OD values for Wallach's previous study, however, the combination of low initial reactivity and the lack of exposure for two months before exposure trials suggests that these birds would have had minimal antibody titres. This is supported by the reduction of titres in the hens housed on concrete in our 2006 study where serum OD values reduced by half after eight weeks on concrete.

This is potentially very important to the game bird industry, as a vaccine that only produces a response in naïve individuals is unlikely to be useful in the game bird industry, or indeed many other circumstances, given the differences in husbandry

practices already discussed and the realities of pathogen exposure in other systems. Any new coccidiosis vaccine will have to be able to produce a response in individuals with a long history of exposure and likely correspondingly high antibody titres whilst weighing the potential costs to mother and offspring.

This then leads on to the second part of this chapter were we examined the effect of environmental exposure on the change in titres. It was hypothesised that CoxAbic treated birds housed on grass would show the highest titres as they would have received a combination of stimuli from the CoxAbic antigens and natural challenge from the coccidia present on the grass within the pens, and PBS treated birds on concrete would have the lowest titres because they were not exposed to any coccidial antigens. However, there was no evidence to support this hypothesis and the only group to significantly increase its titres were PBS treated birds on grass. These birds were not deliberately inoculated with coccidial antigens, but will have picked them up from the environment by ingesting oocysts. This is an interesting effect as it would seem to suggest that there may be a cost associated with the vaccine that manifests as a reduced ability to mount a humoral immune response consequently leading to lower antibody titres.

This could potentially be due to differences in the immunogenicity of the *Eimeria maxima* derived antigens in the vaccine compared to entire pheasant *Eimeria* found naturally in the environment. For example, there could have been a disproportionately large and targeted response to the vaccine antigens, resulting in a reduced response to the naturally occurring infection, whereas the PBS treated birds

on grass were able to mount their usual response to the naturally occurring antigens alone. This could lead to a situation where vaccine treated birds are better primed to deal with the vaccine based antigens from a species of *Eimeria* that cannot infect them, and only able to mount a minimal response to naturally occurring pheasant *Eimeria*. Whereas the placebo (PBS) treated birds on grass are fully able to mount an optimal response against the naturally occurring pheasant *Eimeria*, the only *Eimeria* antigens they have encountered. There is some evidence for this in Figure 4.6 where PBS treated hens on grass showed a significant increase in serum OD values, PBS treated hens on concrete tended to show a non-significant decrease in OD values, and CoxAbic treated birds showed a non significant increase in OD values. CoxAbic treated birds on concrete showed a non significantly higher increase in OD values, and this could potentially be due to the fact that they were not having to mount an immune response against the naturally occurring *Eimeria* as well as the vaccine antigens.

The presence of mould on some of the samples was unexpected as all samples from 2007 were handled and stored in the same way as those from 2006. However, antibodies are perhaps one of the most stable molecules in the blood (Harlow and Lane, 1999) and so it is unlikely that they were denatured through any action of the mould. Furthermore, although the blood samples showed signs of particulate matter, it is highly unlikely that this could have affected the ELISA readings. The ELISA technique relies on two very specific binding stages, firstly where the antibodies in the sera bind to the antigens on the plate, and secondly where other antibodies bind to the serum antibodies bound to the antigens on the plate. In between these steps

there are numerous detergent washing stages designed to remove non-specifically binding molecules, which would remove any mould derived matter. The final step in the ELISA technique requires alkaline phosphatase conjugated antibodies to digest the colourless substrate to yellow ρ – nitrophenol. If no serum antibodies are present then the conjugated antibodies would simply be washed out of the wells as they would not be bound, and the addition of the substrate would have no effect as there would be no alkaline phosphatase enzyme present to digest it, resulting in a colourless solution and a low OD value. This did not happen, and all serum wells showed some sign of ρ – nitrophenol, whereas the blank control wells did not. It is possible that mould derived alkaline phosphatase caused this reaction, however based on the discussion of the exclusion of non-specific binding this seems very unlikely. It is also very unlikely that any of the above effects would have lead to an increase in serum OD values, as any denaturation of antibodies, which is the most likely effect of mould, should lead to a reduction in the OD values. However, to prove that mould could not have affected these samples it would be necessary to run ELISA tests on samples that are known to work, and to then repeat the ELISA tests with aliquots of the same samples that have been allowed to go mouldy. Any effect of the mould on the samples would then be immediately obvious.

The CoxAbic vaccine did not produce a significant immune response, but there was a wide range of naturally occurring OD values in the hens. There was also a non-significant trend for hens with higher body condition scores to have higher serum OD values, and for this relationship to be influenced by the substrate they were raised on. This is shown by the substrate by treatment interaction and near significant substrate

effect when looking at the relationship between change in body condition and OD values. This suggests that individuals that are able to increase their body condition are also able to increase their OD values, especially so on grass. This is contrary to predictions that birds would trade-off immunity against other life history traits and could potentially be a case of the "big house, big car" theory as reviewed by Reznick *et al.* (2000), and as seen in red jungle fowl (Zuk and Johnsen, 2000). The theory behind this is that some individuals are better at accessing and using resources than others. Therefore, although there may be a trade-off between immunity (serum OD values) and body condition there may also be natural variation in the amount of resources available to each individual such that a "super-individual" can have a larger resource pool and alter it's investment in different traits, but still be investing more in each trait than individuals who are less able to utilise, or who have access to reduced resource pools. Based on these results it may only be possible to see life history trait trade-offs when some resource is limited as the variation in resource utilisation and acquisition between individuals masks any other effect (van Noordwijk and de Jong, 1986).

Trade-offs between reproduction and immunity have been studied extensively in other avian systems (Reviewed by Norris and Evans, 2000; Martin *et al*., 2008) and frequently find that increased investment in reproductive effort tends to lead to a decreased ability to mount an immune response (Nordling *et al.*, 1998; Råberg *et al.*, 2000; Ardia, 2005; Boughton *et al.*, 2007) (but see Friedl and Edler, 2005). However, these, and many other studies have tended to use wild populations where it is more likely that some resource such as food or space will act as a limiting factor, thereby

forcing trade-offs between reproduction and immunity, whereas the experimental system used in this study provided *ad libitum* food which may have mitigated any trade-off. The Boughton et al. (2007) study, which was conducted with captive Chinese Quail still found a "reproductive tissue" mediated trade-off between immunity and reproduction. However, the variation in reproductive status was achieved through castration, which may have had significant extra effects on the immune system due to the complicated effects of testosterone throughout the body and across the breeding season.

Futhermore, the previously mentioned studies all used novel antigens to explore trade-offs between immunity and reproduction (for example sheep red blood cells or human diphtheria-tetanus vaccine) whereas our study used antigens from coccidia, which are a common pathogen of pheasants. Studies with antigens that a bird is never likely to naturally encounter are useful at empirically demonstrating the theories behind life history trade-offs. However, it is vitally important that effort is also devoted to looking at these effects in real life systems. This chapter has demonstrated this to some degree by finding no evidence for an effect of the CoxAbic vaccine in pheasants, which is likely to be a result of the difference in rearing systems in the game and poultry industries. This could be confirmed by testing naïve pheasants reared in a coccidia free environment from hatching.

It is also important to consider the ecological basis for trade-offs between reproduction and immunity. Parasites such as helminths often show spring population peaks for a number of reasons including the vernalisation of parasite eggs

necessary to some parasite lifecycles, and increasing sociability of wild bird and mammal populations leading to greater host density. This of course occurs at a time when many organisms are becoming reproductively active, and so are forced to trade-off investment in reproduction against the need to mount immune responses against the increasing parasite population. This also often coincides with an increase in food availability and increasing ambient temperature, meaning a potential rise in energy intake, and a decrease in energy expenditure required to maintain body temperature, allowing for a larger pool of resources to allocate to life history choices. Therefore any trade-offs that are occurring between immunity and reproduction may be masked in the wild by greater food availability and altered energy requirements allowing for greater investment in costly traits such as immunity and reproduction now that the costly energy constraints of limited food and cold weather are removed. In other words, a trade-off may be occurring, but it is occurring from a relatively larger pool of resources such that absolute investment in different traits may not change.

This chapter has not found any evidence for life history trade-offs, but it has again shown the important role of rearing substrate. These results suggest that it is necessary to use naïve birds in order to explore these effects more thoroughly, however this risks producing misleadingly positive results that are not applicable to the industry. This appears to be especially true in the game bird industry given the cosmopolitan nature of coccidia and the life-long exposure that these birds experience as a consequence of modern husbandry techniques.

Chapter 5 - Measuring the effects of male characters on female reproductive effort in Phasianidae

5.1 Introduction

Life history theory states that organisms are constrained by limited time and energy and that investment in one activity reduces the resources available to invest in others (Williams, 1966). Therefore a breeding female is predicted to face decisions about how to trade off investment in current reproduction against future opportunities with the potential for imminent loss of resources or death. Females are therefore expected to adjust their reproductive investment in aspects of reproduction, such as egg size and clutch size, relative to local cues such as resource availability, disease exposure, and seasonality. In biparental systems a female's optimal allocation may also be affected by her mate. For example, he may differ in the resources he provides such as direct resources such as food and indirect resources such as genetic benefits. It may therefore be beneficial to alter investment relative to male attractiveness as this may be a good signal of direct or indirect benefits that the mate could offer, such as better paternal care (Hoelzer, 1989; Wedell and Karlsson, 2003; Johnsen *et al.*, 2005), or genetic benefits (Fisher, 1930; Moore *et al.*, 1997; Gwinner and Schwabl, 2005; Head *et al.*, 2006). These genetic benefits could potentially influence factors in later life such as development of secondary sexual characteristics which may impact on offspring reproductive success (Hamilton and Zuk, 1982; Lindstrom, 1999; Forstmeier *et al.*, 2004; Saino *et al.*, 2007), and hence female life time reproductive success.

In the context of our studies on maternally derived immunity, female allocation decisions could potentially have an important influence on traits of interest. For example, females mated to more attractive males could provision their eggs with more immune factors (Saino *et al.*, 2002a; Saino *et al.*, 2002b), alter the hormonal environment which in turn could affect immunocompetance (Gil *et al.*, 1999), or alter clutch characteristics (Rutstein *et al.*, 2004) which could then potentially influence chick condition or survival (Williams, 1994; Cunningham and Russell, 2000). These are important variables in our studies on the effectiveness of the CoxAbic vaccine so it is important that we have some knowledge of female allocation decisions so that we can either control for them experimentally, or if they are large, account for them statistically. This would then give us more power to explore the costs and benefits of the CoxAbic vaccine on the chicks.

5.1.1. Differential allocation

The adjustment of female allocation in response to male traits is referred to as differential allocation, and was first explored by Burley (1981) in a zebra finch model system. This study found that it was possible to alter male attractiveness independently of other traits such as condition, through use of coloured leg bands. Males with red leg bands were found to be more attractive than those with green leg bands, and females mated with red banded males tended to have higher reproductive success measured as the number of young surviving at least two weeks past fledging (Burley, 1986). This is important as it shows that females are able to alter investment in their current reproductive effort in response to artificially induced male

attractiveness, independent of potentially confounding factors such as a males ability to coerce a female, or more subtle cues about male traits such as condition.

There has since been a large number of papers published in support of this hypothesis in the zebra finch system (Gil *et al.*, 1999; Rutstein *et al.*, 2004; Rutstein *et al.*, 2005b), but also in species with precocial offspring such as wildfowl (Cunningham and Russell, 2000) and game birds (Chinese painted quail) (Uller *et al.*, 2005). These studies often find that females mated to more attractive males invest more in some aspect of investment in their offspring than when mated to less preferred males, for example hormones or nutrients, leading to greater offspring survival, greater hatching weight, or hatchability.

The potential role of differential allocation in gamebirds has previously been explored in Chinese painted quails (Uller *et al.*, 2005). This study found that females mated to males with larger badges produced larger, but not more eggs. However, this study used quails of both sexes that had been treated *in ovo* with testosterone (4-androsten-17β-ol-3-one) during previous experiments on the effects of testosterone on the interaction between immunity and ornamentation (Uller *et al*., 2006) and growth rate and immunocompetence (Andersson *et al.*, 2004). Administration of *in ovo* testosterone was shown to reduce testis size and mass, but did not affect badge size. Furthermore females laid smaller eggs for testosterone treated males regardless of other traits and females who received *in ovo* testosterone themselves produced smaller eggs regardless of the male they were mated with.

Due to this manipulation it is difficult to assess the effects of testosterone on male traits that females may respond to, and to assess the impact of testosterone on the female's basis or ability to alter her investment in different breeding attempts. For example, increased *in ovo* testosterone levels could have behavioural or physiological effects on males or females, leading to different levels harassment of varying ejaculate characteristics. Nevertheless, these studies suggest that differential allocation can occur in the Chinese painted quail, and future studies would benefit from using unmanipulated offspring to see if differential allocation occurs, as theory would predict in unmanipulated birds.

Contrary to the differential allocation hypothesis is the 'compensation hypothesis', put forward by Gowaty (Gowaty, 1996; Gowaty and Buschhaus, 1998; Gowaty, 2003; Gowaty, 2008). The compensation hypothesis predicts that females should increase reproductive effort in response to matings with non-preferred partners, or matings under sub-optimal conditions, in order to compensate for decreased offspring viability. A more recent paper (Gowaty *et al.*, 2007) attempted to show this experimentally, but rather than focusing on a single system this paper reports a number of small, and ultimately ambiguous studies, in a number of different systems. However, evidence for the compensation hypothesis has also been documented in zebra finches (Bolund *et al.*, 2009), where females mated with attractive males (as measured by extra-pair matings and offspring rearing success) produced smaller (by volume) eggs with less carotenoids in the yolk.

Differential allocation theory is based on current investment being costly to future reproductive attempts, and although it is relatively easy to prove that maternal allocation does occur, and can be in response to the mate, its effects on future reproductive success have rarely been shown. One example is the *Rana lessonae-Rana esculenta* (LL-LR) waterfrog complex (Reyer *et al.*, 1999) where females were able to adjust the amount of eggs they released in response to the male. This was shown to have an effect on subsequent clutch size. For example, when forced to mate with a non-preferred male females released less eggs, but were able to release more during subsequent matings with preferred males.

Many studies rely on male attractiveness as the primary cue in differential allocation studies, but even here there are some studies that have found no evidence for or dispute the evidence for differential allocation (Witte, 1995; Mazuc *et al.*, 2003), or find no evidence that it is occurring in response to mate attractiveness (Johnsen *et al.*, 2005; Michl *et al.*, 2005; Uller *et al.*, 2005; Nakagawa *et al.*, 2007b). However, there are many more studies that have shown that mate attractiveness can be directly related to female reproductive investment (Burley, 1988; de Lope and Møller, 1993; Cunningham, 2003; Limbourg *et al.*, 2004; Osorno *et al.*, 2006; Loyau *et al.*, 2007). Sheldon (2000) has provided a good review of the differential allocation hypothesis that focuses on the direct consequences of male attractiveness on female reproductive effort. However, it is important to note that male attractiveness can be more than just an arbitrary signal, and can potentially act as a signal for non-genetic factors such territory quality or paternal care (Preault *et al.*, 2005), and genetic factors such as increased immunity.

This chapter will examine female reproductive investment, such as egg size and number, in pheasants and Chinese Painted Quail in response to male traits that have been shown to affect female preferences. This will be achieved by collecting eggs of individuals mated to males with measured characteristics and then comparing their egg laying characteristics when mated with a different male.

5.2 Experiment 1 (Ring-necked Pheasant)

5.2.1. Sexual selection and differential allocation in the pheasant

Pheasants are a highly sexually dimorphic species and there are a number of potentially important traits involved in sexual selection. Some of the most important traits for female choice include aspects of territory holding such as territory fidelity (Goransson, 1984; Ridley and Hill, 1987; Goransson *et al.*, 1990) and quality (Grahn *et al.*, 1993), as well as male condition and dominance (Ridley and Hill, 1987; Mateos and Carranza, 1995).

Male dominance is determined through male-male interactions (Collias and Taber, 1948; Collias and Taber, 1951; Hunt *et al.*, 2009), though it can be estimated from male traits such as spur length (Koubek and Hrabe, 1984; Goransson *et al.*, 1990) age (Mateos and Carranza, 1996), and body condition (Koubek and Hrabe, 1984; Briganti, 1992; Grahn and von Schantz, 1994; Mateos and Carranza, 1996), all of which are important in territory acquisition and retention (Collias and Taber, 1951; Burger, 1966; Lachlan and Bray, 1976; Ridley, 1983). Therefore traits informative to

male-male contests may also have indirect effects on female choice through their action on territory acquisition, and may also be directly informative for female choice, for example spur length (von Schantz *et al.*, 1989; Goransson *et al.*, 1990; Mateos and Carranza, 1995; Mateos and Carranza, 1996).

In an experimental setup it is neither practical nor ethical to release male pheasants into a large area and allow them to establish natural territories and dominance hierarchies unrestrained as this is likely to lead to injuries, or even deaths. However, there are numerous ways to measure dominance experimentally, such as outcomes from antagonistic encounters or avoidance and submissive behaviour. In *Gallus gallus* a recent study found that in a linear hierarchy all of these dominance indices were highly correlated with crowing rate (Bayly *et al.*, 2006). Furthermore crowing rates have been shown to strongly correlate with male dominance behaviour in pheasants (Collias and Taber, 1948; Taber, 1949). Crowing, and it's associated flapping display in pheasants is usually restricted to territory holding males (Taber, 1949; Burger, 1966). However, males of similar dominance will often respond to this with a single coughing call with no associated flapping.

5.2.2. Materials and methods

This experiment first examined the relationship between male traits that have been shown to be important to female choice, such as secondary sexual characteristics, dominance and condition. Then the effect of these traits on female allocation decisions was explored.

5.2.2.a. Assessing male traits

Six males were communally housed in a single 10’ x 10’ pen to encourage formation of a dominance hierarchy. The males were fitted with spectacles (Figure 5.1) to reduce directed aggression such as feather pecking and attacking with spurs, but allow for normal dominance behaviours such as crowing. Observational studies of male dominance were performed before and after the first mating period, no more than one hour after sunrise, when male-male interactions are most common (Kimball, 1949; Gates, 1966). Crowing rate, responding calls, intimidation behaviour and sparring contests were all recorded during the 30 minute observation.

The inflation of wattles was assessed subjectively on a scale from 1 to 3 where 1 represented no wattle inflation and 3 represented full wattle inflation where the wattle and combs extended above the top of the skull and below the neck (Figure 5.2). An index was used as it was found that directly measuring wattle size or attempting to measure wattle size from photos was highly unrepeatable as when captured and handled all males deflated their wattles. The length of the tail and the length of the spurs were also measured as both of these have been shown to have positive effects on male reproductive success.

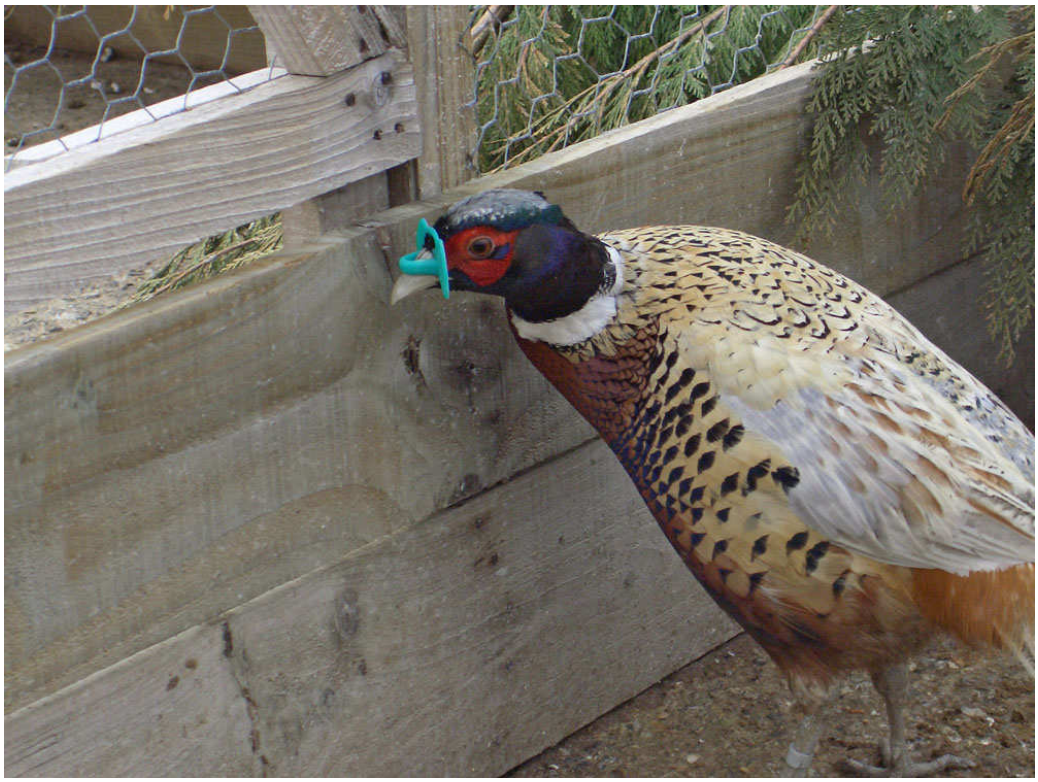

Figure 5.1: A pheasant cock fitted with “spectacles” in the experimental pens

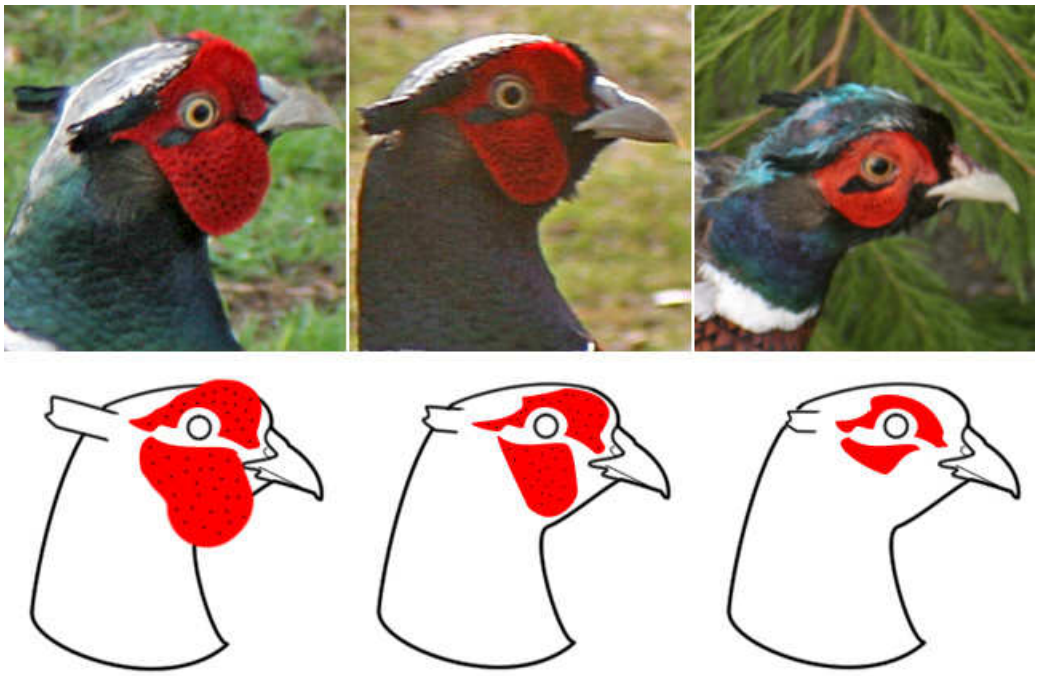

Figure 5.2: Variation in the secondary sexual characteristic of male pheasants showing how the ear tufts, wattles and combs can vary between individuals. These images are aligned left to right in order of descending wattle score, such that the left most individual would be scored a 3, and the right most individual a 1.

5.2.2.b. Measuring female reproductive investment in response to male traits

Thirty six females were housed in six harems of six in 10’ x 10’ pens on concrete. This is a small harem, however six hens is still within the natural range of harem sizes (3 - 18 hens; Ridley and Hill, 1987; Goransson *et al*., 1990). All birds were treated with Baycox (active ingredient Toltrazuril; Bayer Animal Health), an anti-coccidial drug, before the experiment to remove any potentially confounding effects of variation in coccidiosis infection levels on reproductive effort. All birds were given access to *ad libitum* feed (Pheasant Breeder Pellets, Gamekeepa Feeds) and water via nipple drinkers (Quill Productions Ltd). Each pen was equipped with two areas of heavy conifer brashing (piled branches of *Cupressocyparis leylandii*) a laying box (Quill Productions Ltd) and a raised 1’ x 4’ mezzanine area. Additional enrichment was provided by suspending CDs in the pen and tying multiple strands of bailer twine to the cage sides. Weight and tarsus length was measured for all birds and used to provide a condition index for each female by plotting a regression line through a plot of weight against tarsus. The residuals were used as the individual condition scores. See chapter two methods for more information. All birds used in this experiment were one year old and this was their first breeding season.

Eggs were collected from each harem pen for two weeks and weight and volume of all eggs were calculated as previously described. These eggs provided a baseline measure for egg characters when no males were present. After two weeks had passed one male was introduced into each harem pen at random. Eggs were collected and measured for a further two weeks, after which time the males were removed and eggs were collected for another two weeks with no male present. This period was to allow

for sperm clearance so that all subsequent eggs could only be sired by the second male. Following this a different male was introduced, and eggs were collected for a final two weeks. Eggs were therefore collected from each female group under a high and low rank treatment with treatment effects equally distributed between first and second clutches.

5.2.3. Statistical analyses

The relationships between male traits and between male traits and female reproductive measure were analysed with Spearman rank correlations. It was not possible to assign eggs to specific females and so each harem pen (containing six females) was treated as the independent unit throughout these analyses.

5.2.4. Results

5.2.4.a. How do different male traits relate to each other?

There were no observations of "intimidation behaviour" and only a single "sparring contest" was observed between the highest crowing and third highest crowing male. Crowing rates were consistent with those recorded by Taber (1949) in wild pheasants and ranged from 0 to 5 crows per 30 minutes. Furthermore, crowing rates were repeatable across the highest and lowest ranked individuals (ranked by cumulative crowing frequency), however it was more variable for middle ranked individuals. Therefore this study will use the cumulative crowing rate which ranged from 0 to 16 (Table 5.1). There was no evidence for any significant correlations between any of the male traits (Table 5.2). However, although the correlation between crowing rate

and wattle score was non-significant (ρ = 0.772, p = 0.072), the effect was in the expected direction (Figure 5.3) with higher crowing males tending to have higher wattle scores. This sample size (6) had approximately 16.5% power, the sample size would need to be increased to 20 to achieve 80% power and 30 in order to achieve 95% power with the effect size and standard error observed.

5.2.4.b. How do male traits affect female reproductive investment?

The relationship between male traits and total number of eggs laid and total reproductive effort was explored using mixed models with the number of eggs laid, or total reproductive effort as the dependant variable and male traits as fixed effects. These found a negative effect of male spur length (χ^2_1 = 13.73, p = < 0.001) and male weight (χ^2_1 = 162.53, p = < 0.001; Figure 5.4) on the number of eggs laid and a negative effect of male weight on female total reproductive effort (χ^2_1 = 35.93, p = < 0.001) with no significant effect of spur length (χ^2_1 = 3.22, p = 0.073). However, there was a significant positive effect of male weight (χ^2_1 = 19.56, p = < 0.001; Figure 5.4) and average spur length (χ^2_1 = 3.97, p = 0.046) on average egg weight.

Table 5.1: **The male crowing rates (per 30 min) and the total crows over all observation periods**

Cock	Crow Rate (per 30min)				Total
1	3	5	1	2	11
2	0	0	0	0	0
3	0	0	1	1	2
4	3	3	3	4	13
5	4	4	4	4	16
6	0	0	5	2	7

Table 5.2: Spearman rank correlation matrix showing the relationship between the measured male characteristics

	Weight	Condition Index	Average Spur Length	Wattle Score	Tail length
Crowing rate	ρ = -0.029, *p* = 0.957	ρ = -0.486, *p* = 0.329	ρ = -0.486, *p* = 0.329	ρ = 0.772, *p* = 0.072	ρ = 0.429, *p* = 0.397
Tail Length	ρ = 0.086, *p* = 0.872	ρ = -0.486, *p* = 0.329	ρ = 0.543, *p* = 0.266	ρ = -0.093, *p* = 0.861	
Wattle Score	ρ = -0.093, *p* = 0.861	ρ = -0.062, *p* = 0.907	ρ = -0.648, *p* = 0.164		
Average Spur Length	ρ = 0.086, *p* = 0.872	ρ = 0.086, *p* = 0.872			
Condition Index	ρ = 0.657, *p* = 0.156				

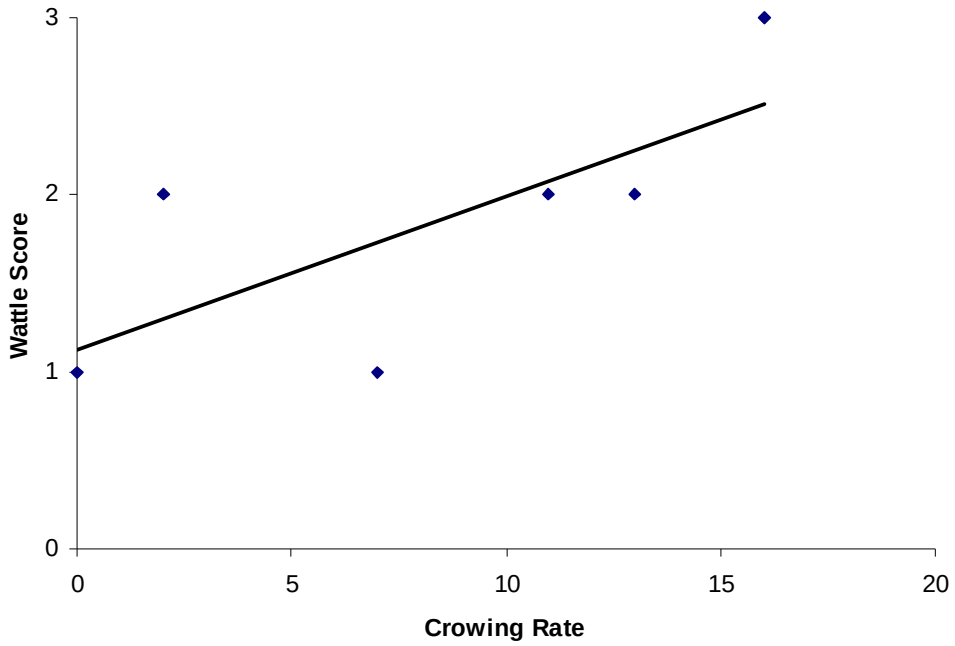

Figure 5.3: The relationship between crowing rate and wattle score with fitted Pearson's correlation ($\rho = 0.772$, $p = 0.072$)

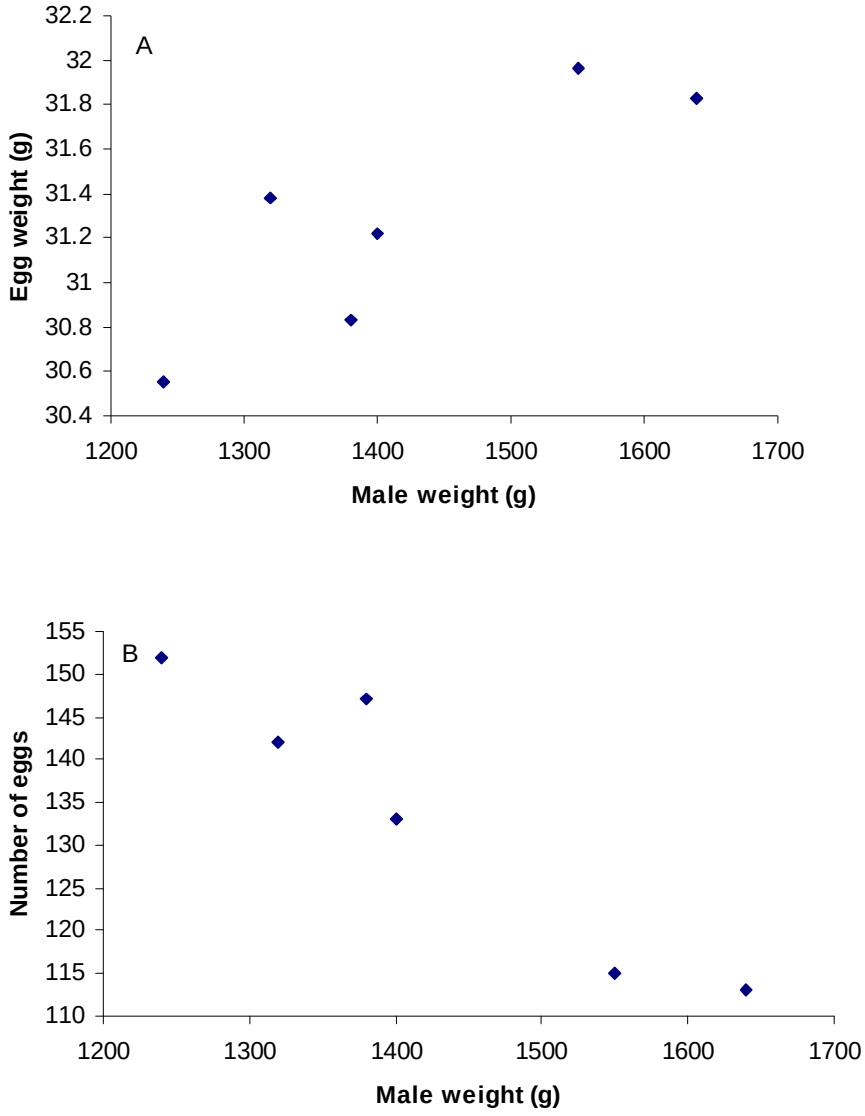

Figure 5.4: The effect of male weight on mean egg weight (g) (A) and number of eggs laid (B)

5.3. Experiment 2 (Chinese Painted Quail)

This section aims to examine the effect of male badge size, a trait considered to be under sexual selection, on female reproductive investment in the Chinese Painted Quail. The results presented here combine data from a series of student experiments with Chinese Painted Quail that collected data on clutch characteristics when individual females were mated to males that varied in badge size. Investment relative to male morphometric traits was also investigated.

5.3.1. Sexual selection in the quail

The Chinese painted quail (*Coturnix chinensis*) is a socially monogamous bird of the family Phasianidae. This species is the smallest in the genus and is sexually dimorphic with the males having a distinct black and white throat badge. There is a high degree of recorded mate switching in this species (Johnsgard, 1988), as there is within the genus as a whole (Rodriguez-Teijeiro *et al.*, 2003), and like other Phasianidae, this species is capable of producing several clutches a year with different mates. This means that differential allocation has the potential to be biologically important in this system. Finally, this species has a long history in aviculture and can produce several clutches in quick succession with different males in captivity. This combination of traits make the Chinese painted quail ideal for laboratory based experiments of the differential allocation hypothesis.

5.3.2. Methods

Two replicates were conducted in which two clutches of eggs were collected from each female, one fertilised by a large badged male and one fertilised by a small badged male with the order in which they received these males equally distributed between treatments.

In the first replicate thirty unrelated birds (fifteen males, and fifteen females) were individually marked with orange numbered leg rings, weighed (to the nearest 0.01 grams) and their tarsus length measured (to end of longest toe, to the nearest 0.1mm). Measurements of the tarsus length were taken by two individuals to check repeatability. The measurements of tarsus and weight were used to generate a residual condition index for each bird by plotting a linear regression of weight against tarsal length, fitting a best fit line and calculating individual residuals from the regression line. The area of the males badge was calculated by photographing males held against a plastic screen at a fixed distance from the camera of 13cm. Two rulers were placed at right angles next to the screen to allow for accurate measurement. The males were then held against the screen with the throats exposed. Photoediting software (GIMP 2.61) was used to calculate the area of the male badges in pixels, which was then transformed to square millimetres using the rulers to calculate a correction coefficient. Each area measurement was repeated twice and used to calculate an average.

Prior to the experiment all birds were housed in two single sex communal cages (2250x450x400m) with woodchip bedding, a shelter area, a sand bathing area,

foliage, *ad libitum* food (Two parts finch food, one part EMP mix and additional Nutrobal calcium balancer (Vetark Professional)) and water and two live meal worms per bird per day. The aviary was maintained at a 14hr light – 10hr dark photoperiod with ambient temperature set at 21^{o}C.

Females were handfed (to prevent use in courtship behaviour) four mealworms per day and their seed mix was supplemented with Prosecto Insectivorous TM (J.E. Haith Ltd) to provide extra protein. Grit was continually available to ensure females had sufficient calcium for egg laying. At the start of the experiment females were housed with their assigned male in pairs, mating was allowed to proceed naturally and females were allowed to lay eggs for two weeks. All eggs were removed daily to be weighed, measured and returned to the nest to encourage natural laying behaviour. After the two week period all individuals were returned to their communal single sex cages for one week to allow for sperm clearance, which lasts on average 6.3 days in the Japanese Quail (Sittmann and Abplanalp, 1965). This week was also to allow for carryover effects, where females may 'set' their investment in relation to the first breeding attempt which could affect allocation decisions in subsequent breeding attempts even if the attractiveness of the male is altered (Charalambous *et al.*, 2003; Rutstein *et al.*, 2004). Each female was then housed with a different male of opposite rank and eggs collected for a further two weeks. The order in which females received a large or small badged male was equally distributed between clutches.

Egg volume was calculated using a species specific volume coefficient (K_v; Hoyt, 1979):

$V = K_v \cdot LB^2$

Where length (L) and breadth (B) are the maximum dimensions of each egg to the nearest 0.1mm, and $K_v = 0.51$. The total volume of all eggs laid per female per clutch was used as total reproductive effort in the analysis of the effect of male traits on female allocation decisions.

In the second replicate the above experiment was repeated with ten males and ten females, unrelated to those used in the previous experiment. No tarsus lengths were collected from this experiment so it was not possible to include male condition index in subsequent analyses.

5.3.3. Statistical analyses

The relationship between male traits was analysed using a Pearson correlation matrix and the effects of male traits on female reproductive effort were examined using linear mixed models. Aspects of female investment traits (egg volume, egg weight and number of eggs) were used as dependant variables in a series of linear mixed models with male traits (badge area and weight), experiment and mating period (1 or 2) as fixed effects and female as a random effect. Entering mating period as a fixed effect allowed us to examine the consistency of females in the allocation decisions. Clutch size was entered as an additional covariate when examining egg size (weight and volume) to control for trade-offs between egg size and number.

5.3.4. Results

5.3.4.a. What is the relationship between the different male traits?

Badge size in the Chinese painted quail is predicted to be a condition dependant trait, therefore we expected to see a significant correlation between the body condition index and badge area. There were no significant correlations between the measured male traits (Table 5.3). However, there was a non-significant but positive trend between badge area and both male weight ($r = 0.471$, $p = 0.076$; Figure 5.5) and condition ($r = 0.464$, $p = 0.081$; Figure 5.5).

Table 5.3: Pearson correlation matrix showing the relationship between male traits. n =15

	Badge area	Weight	Body condition
Badge height	r = 0.188 p = 0.502	r = 0.340 p = 0.215	r = 0.070 p = 0.805
Body condition	r = 0.464 p = 0.081	r = 0.889 p = < 0.001	
Weight	r = 0.471 p = 0.076		

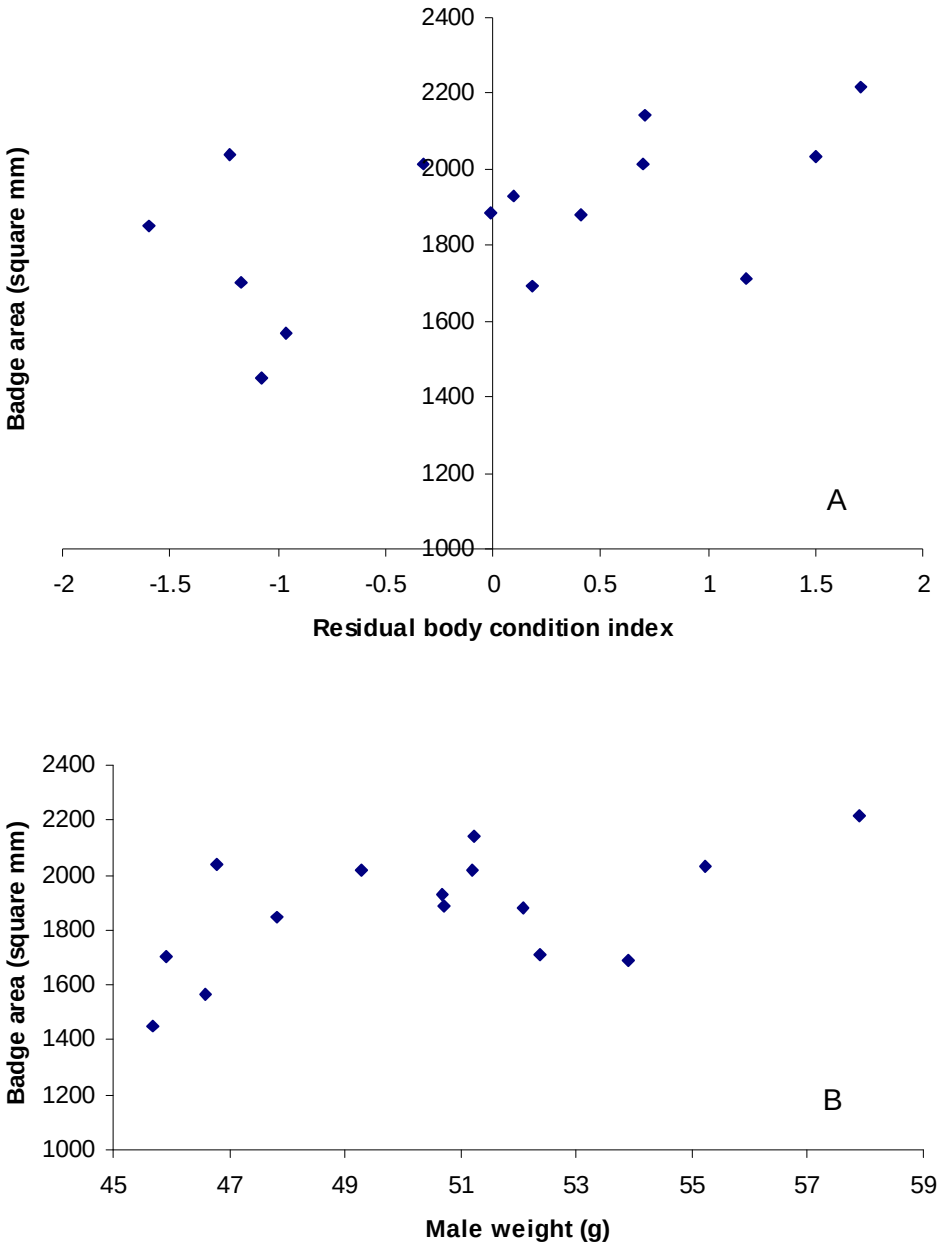

Figure 5.5: The relationship between male condition and badge area (A) and male weight and badge area (B)

5.3.4.b. How do male traits affect female reproductive investment decisions?

Badge area had a significant effect on the volume of eggs laid, with females laying smaller eggs for males with larger badges ($\chi^2_{1,404}$ = 4.74, p = 0.029; Figure 5.6). However, male weight ($\chi^2_{1,402}$ = 1.43, p = 0.232), experiment ($\chi^2_{1,404}$ = 0.13, p = 0.723) and mating period had no measurable effect on egg volume. Badge area was found to have the same effects on egg weight, with females mated to males with larger badges producing lighter eggs ($\chi^2_{1,399}$ = 6.34, p = 0.012; Figure 5.6). However, again there was no evidence that male weight ($\chi^2_{1,396}$ = 0.20, p = 0.655), experiment ($\chi^2_{1,396}$ = 0.40, p = 0.529) or mating period ($\chi^2_{1,398}$ = 2.70, p = 0.100) had any effect.

We examined the effects of male traits on total reproductive effort across both experiments in order to assess any trade-offs between egg size and number of eggs. The effect of male traits on the trade-off between egg size and number has already been partially examined by including clutch size in the earlier models, however total reproductive effort is an alternative way of exploring this relationship. Male weight was found to have a significant effect on total reproductive effort, with females investing less when mated to heavier males ($\chi^2_{1,42}$ = 5.77, p = 0.016; Figure 5.7). However, there was no evidence for any effect of male badge area ($\chi^2_{1,39}$ = 0.33, p = 0.564), mating period ($\chi^2_{1,39}$ = 0.71, p = 0.401) or replicate ($\chi^2_{1,39}$ = 1.29, p = 0.256) on total reproductive effort.

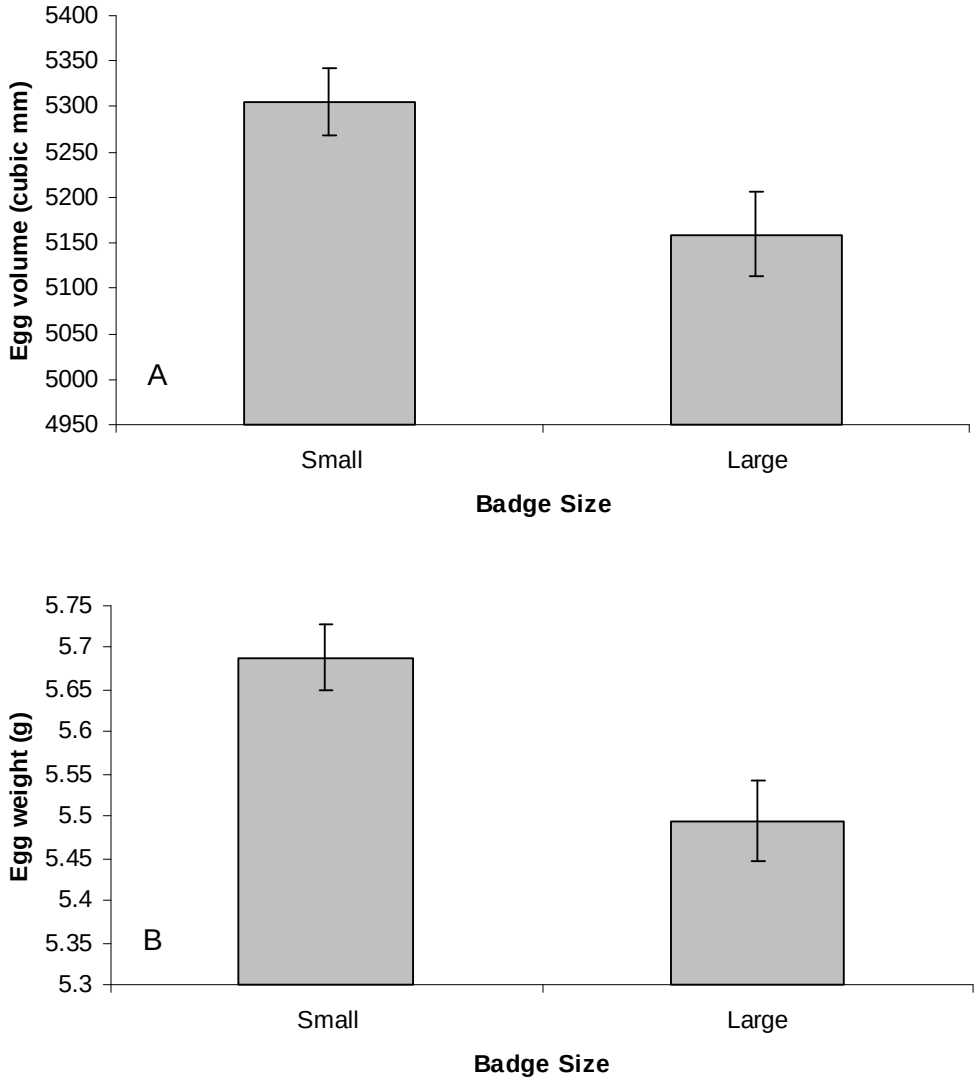

Figure 5.6: The relationship between male badge area and mean egg volume (±se) (A) and weight (±se) (B). This figure shows mean egg weight assigned to a categorical badge size relative to the mean badge area, to aid clarity.

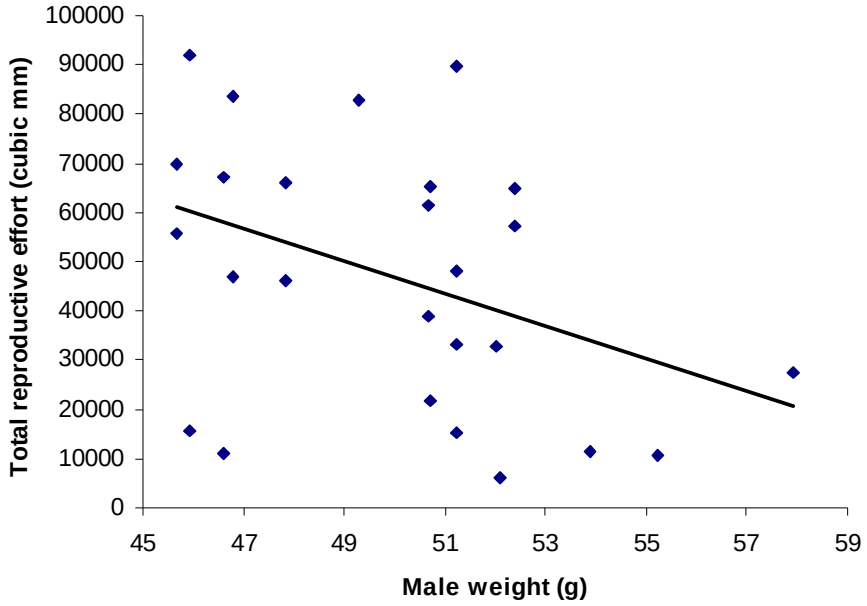

Figure 5.7: The relationship between male weight and total reproductive effort in the Chinese Painted Quail with trend line. Removal of individuals weighing over 55g does not affect the significance of the relationship

5.4. Discussion

This chapter found that male characters can have a significant effect on female reproductive investment. More specifically male weight and average spur length had a negative effect on total reproductive effort in both pheasants and quail, and male badge area had a negative effect on egg weight and volume in quail. There was no significant evidence for any relationship between male traits in either species. However, there were non-significant trends for male pheasants with higher crowing rates to also have higher wattle scores, and for heavier and better conditioned male quail to have greater badge areas and larger badge heights. The lack of a significant effect could be the result of small sample sizes, the interaction of a number of male traits, or simply the lack of a relationship.

The pheasant experiments found that females laid significantly less, but larger eggs when mated to heavier male pheasants and males with larger spurs, and female quails laid smaller (volume) and lighter (mass) eggs for males with larger badges. This is in contradiction to the work of Uller *et al*. (2005) who found female quails laid heavier eggs for males with larger ornaments. Many of the shortcomings of this experiment, and in particular the *in ovo* treatment with testosterone, have already been discussed in the introduction.

The exact mechanism for the observed effect is unclear, and although these results fit the model of the compensation hypothesis there are alternative explanations, such as preferred males may normally be associated with greater direct benefits thereby reducing the need for females to invest to such a great extent. In painted quails for

example, males normally provision and courtship feed the females – if large badged males can feed the offspring more than smaller badged males then females could feed less with no net cost to offspring. Alternatively, increased harassment of females by larger males could reduce the female's ability to invest in her reproductive attempt, by reducing the time available to the female to feed, or increase stress due to the inability of the female to escape. This has been shown to have a wide range of costly effects on the female (Reviewed by Clutton-Brock and Parker, 1995), including increased predation risk (Rowe, 1994), forced movement of the female (Sundaresan *et al.*, 2007; Low, 2008), generalised reductions in reproductive fitness (Ronn *et al.*, 2006; Gay *et al.*, 2009) and even injury (Pizzari, 2001) or death (Leboeuf and Mesnick, 1991; Reale *et al.*, 1996). As previously stated, the compensation hypothesis predicts that females should increase investment in the current reproductive attempt when mating under sub-optimal conditions, for example, in a poor environment or with a non-preferred male. This increased investment then "compensates" for the costs of mating under suboptimal conditions, such as reduced food availability for the offspring or "poor genes" from the male. This is an attractive and somewhat intuitive hypothesis, however this hypothesis, and indeed many others place too great a stress on the costs or the benefits of a mechanism rather than exploring both. For example, it is also possible that females actually decrease investment under optimal conditions, such as in food rich environments or when mated to a preferred male, as the positive effects of the environment, or the "good genes" from the preferred mate reduce the burden of investment on the female. This is a similar effect to that seen by Russell *et al.* (2007;

2008) where female superb fairy wrens (*Malurus cyaneus*) showed a reduction in egg investment and maternal feeding rates in the presence of helpers.

More recent work on the compensation hypothesis suggests that compensatory investment in a reproductive attempt is more likely when there is little effect of parental investment on offspring quality or baseline survival (Harris and Uller, 2009), a low chance of meeting a higher quality mate in the future, or a low level of reproductive skew (Bolund *et al*., 2009). Reproductive skew is a measure of the effect of social and ecological factors on how reproductive output is spread throughout a population. Therefore offspring in populations with a low reproductive skew from low quality matings can still be expected to successfully reproduce. As a socially monogamous species the Chinese painted quail fit these assumptions, however pheasants do not. Due to the hierarchical structure of pheasant communities there is a high degree of reproductive skew (von Schantz *et al.*, 1989; Mateos, 1998) with 15%-60% of males having little reproductive success (Lachlan and Bray, 1976; Ridley and Hill, 1987). Females can move between territories and visit several males, so it is possible that there is a chance of meeting a better quality mate, furthermore females can abandon their current reproductive attempt in response to environmental factors (Einarsen, 1945; Persson and Goransson, 1999) and it is possible that encounters with a higher quality male may induce nest abandonment as well.

Based on the hypotheses put forward by Bolund *et al.* (2009) pheasants should exhibit differential allocation. The fact that there is evidence that potentially shows compensatory investment may be a result of the captive environment. The pheasant experiment found a significant relationship between male weight and female

allocation. However, there was no relationship between male weight and other male traits that might be expected to influence female allocation decisions, such as wattle size, spur length or dominance suggesting that females based their allocation decisions on male weight alone, and other sexual characteristics were being ignored. The lack of a relationship between male traits is in contrast to a previous study (Papeschi *et al.*, 2003) that found a significant relationship between dominance (as determined by behavioural observations of wild tagged birds) and wattle size (recorded as "closed", "half open", or "erect"). It is possible that by separating males and actively preventing aggressive male interactions our experiment has removed the normal dominance processes and so characters usually associated with dominant males have become uninformative. For example, a male with large spurs may win more antagonistic encounters (Davison, 1985; Mateos and Carranza, 1996) and so secure a large territory (Collias and Taber, 1948; Collias and Taber, 1951; Grahn *et al.*, 1993) with greater access to food and so a greater ability to increase condition and invest more in traits that are attractive to females, but with access to *ad libitum* food and segregation of males all males can invest in attractive traits without the costs of male-male interactions, and without limited access to food. However, there may be differences in resource acquisition and allocation as discussed in chapter 4 that could lead to differences in perceived attractiveness even under an *ad libitum* feeding regime.

These results also suggest that under these conditions wattle size is not condition dependant as we found no relationship between wattle size and condition or weight. It has been shown that protein availability in early growth can affect wattle size and

colouration in later life (Ohlsson *et al.*, 2002; Ohlsson *et al.*, 2003) and that young adult males in better condition are more able to increase their wattle size when given supplemental carotenoids (Smith *et al.*, 2007). In the wild condition, and especially protein availability may be important in wattle development, whereas carotenoids, and their interaction with condition, are important in the deployment and maintenance of wattles in later life. However, this effect is masked in captivity by provision of *ad libitum* food which could potentially alleviate energetic demands.

However, the quail data found a significant correlation between badge area and male weight and condition, providing evidence that this may be a condition dependant trait. This has been observed in blue tits (Griffith *et al.*, 2003), where crown UV colouration was shown to be a good predictor of overwinter survival, and as seen as a weakly positive relationship in a recent meta analysis of house sparrow badges (Nakagawa *et al.*, 2007a). The differences in condition are likely to be due to differences in resource acquisition as all birds were fed *ad libitum* and kept free from disease, which should remove any burden of immunity and allow optimal investment in life history traits.

Previous work on Chinese painted quails (E. Enright, 2005 & P. Boulcot 2007, unpublished data) found that there was no relationship between male badge size and social dominance, which is in contrast to the Nakagawa House Sparrow meta-analysis (Nakagawa *et al.*, 2007a) and other studies (Senar *et al.*, 1993; Chaine and Lyon, 2008). However, male-male interactions may only be important in wild situations where male-male conflict has direct effects on access to females or food.

This therefore may be another example of "big house, big car" (as discussed in Chapter 4). For example, all males had access to a large resource pool (*ad libitum* food); therefore all males had the ability to increase their investment in sexual characteristics such as badges.

It is important to consider that these experiments were conducted in controlled captive populations, and therefore may not reflect the true outcome in natural wild populations. This is especially true in the pheasant system where dominance in the wild, and hence territory possession and harem size is determined by male competition including highly aggressive antagonistic encounters which we could not replicate in these experiments. As mentioned in the introduction this potentially has important effects when looking at mate choice experiments in captivity as aspects of male attractiveness, such as wattle size and social dominance are often determined in the wild by the outcomes of antagonistic encounters (Fröberg and Helgée, 1985; Ridley and Hill, 1987; Mateos and Carranza, 1997).

The sex of the collected eggs was unknown and so it is possible that rather than increasing egg investment *per se* females were actually altering the sex ratio. Altering the sex ratio in response to male attractiveness has been observed in a number of species (Griffith *et al.*, 2003; Rutstein *et al.*, 2005b; Korsten *et al.*, 2006) where females tend to increase the proportion of the most "expensive" sex or the sex with the greatest reproductive potential. Males are usually the more expensive sex and so sex allocation experiments have usually observed increase proportions of males when mated with attractive males (However see Rutstein *et al.*, 2005b where

females increased the proportion of female offspring in response to attractive mates). However, the females in our experiments produced lighter eggs, which could indicate an increased proportion of female offspring, for example as seen in house sparrows (Cordero *et al.*, 2000). In the future any presence of sex ratio allocation could be confirmed with molecular sexing of the embryo. Though it is important to note that females can potentially alter sex ratio directly by producing more eggs of particular sex (primary sex ratio control), or through differential deposition of hormones and nutrients to eggs of different sexes (Saino *et al.*, 2003b; Groothuis *et al.*, 2005; von Engelhardt *et al.*, 2006), potentially leading to sex-based differences in survival or hatchability (secondary sex ratio control) of the different sexes.

These results are also potentially important for industry. For example, the negative effect of male weight on total reproductive effort could have implications for production, especially if compounded with the negative effects on egg characteristics of rearing on concrete, as seen in chapter 2. Although it wasn't measured in these experiments it is also possible that females could also alter their transfer of immune components in response to male characteristics. For example, as seen recently in a number of passerine species (Gilbert *et al.*, 2006; Williamson *et al.*, 2006; Torok *et al.*, 2007). Again, coupled with the negative effects of rearing on concrete this could have long lasting detrimental effects in the offspring.

This chapter has shown that both Chinese painted quails and ring necked pheasants have the potential ability to differentially allocate resources to different breeding attempts with males of differing attractiveness. However, the pattern of this

investment more closely matches that of the compensation hypothesis, and not the differential allocation hypothesis, which in the case of the quail experiments is in direct contrast to that of previously published work (Uller *et al*., 2005). Given the stark differences between these studies further experiments should be encouraged with unmanipulated individuals, with particular attention paid to the different aspects of female reproductive investment, such as egg weight and volume and clutch size and additional male traits, such as weight and condition, as well as badge size.

Chapter 6 - Discussion

The main aim of this study was to investigate the costs and benefits of maternal resource allocation in response to vaccination on mothers and their offspring. This study consistently found a significant cost associated with being raised on concrete, rather than grass, and a significant effect of cohort. However, this study has found that the CoxAbic vaccine does not produce a measureable immune response over and above environmental exposure in pheasants. Finally this study found evidence which is potentially consistent with the compensation hypothesis from three independent studies (two in the Chinese painted quail and one in ring necked pheasants) where females mated to unattractive males laid larger or less eggs than when mated to more attractive males as measured by body size or badge size.

The possible reasons for the lack of a vaccine effect in field studies have been discussed in the relevant chapters (Chapters 2 and 4). However, in summary it is possible that the lack of evidence for a vaccine effect is due to differences in the rearing practices of the game and poultry industries. The main difference being that the poultry industry raise their birds at much higher densities, but with much stricter cleanliness and hygiene controls, for example regularly cleaned and disinfected concrete pens. The game industry often raises birds outside on grass that may have been used for rearing for many years. Therefore game birds are likely to be exposed to a larger spectrum of pathogens from a younger age, including *Eimeria*, whereas poultry are often naïve, and therefore are likely to have a lower background response to the antigens and to produce a much more detectable response to the vaccine

antigens. It is also possible that the primary immune response from naïve birds would actually be lower than the memory response from previously exposed birds, unless the antigens used in the vaccine are different from the antigens the previously exposed birds naturally responded to. This is possible since although the vaccine antigens are likely to be conserved across species, it is possible that the birds could have responded to other antigens present on different life stages of the parasite.

Due to the differences in rearing practices future studies would benefit greatly from two positive control groups; one in which pheasant chicks were raised from hatching under coccidia free conditions and treated with the vaccine as directed and a second where the same vaccine batch was used on a clean chicken flock reared under identical conditions. If no vaccine effect is seen in the two control groups then this may indicate a problem with the vaccine, and if an effect is seen in the chickens but not the pheasants then the vaccine is unsuitable for use in pheasants because of reasons other than rearing practices.

Most studies of the CoxAbic vaccine to date have not looked at the direct immunological effects of the vaccine, rather focusing on its ability to reduce oocyst excretion (a proxy measure of pathology). A more thorough examination of the immunogenic properties of the CoxAbic vaccine, and especially its constituents (Affinity purified gametocyte antigens and Freund's incomplete adjuvant) should be conducted in the species that have so far trialled the vaccine and under coccidia free conditions, as discussed above. Furthermore other measures of pathology such as changes in weight and condition should be considered.

The largest and most consistent effect seen throughout these studies was that of substrate. Birds raised on grass, rather than concrete were in better condition, weighed more, laid more but not larger eggs and produced chicks that were in better condition after challenge with live coccidia. It is likely that there are a number of causes behind these results ranging from environmental enrichment to constant exposure to low levels of environmental coccidia.

All pens were equipped with the same amount of furniture (platforms, hanging ropes, laying boxes, areas of brashing and grit trays) and in the same arrangements. However, the grass pens are likely to have been perceived as a more enriched environment for several reasons that have already been discussed, such as increased stimulation to forage, and therefore less likely to cause stress (Rodenburg and Koene, 2007). For example, the availability of natural invertebrates such as crane flies, ants and butterflies provided additional protein to the birds as well as activity. Furthermore the grass pens allowed the birds to dust bathe which is a natural behaviour observed in many bird species that helps to maintain feather condition (Healy and Thomas, 1973; Vanliere and Bokma, 1987). Birds reared in an environment where they can dust bathe and then subsequently moved to an environment where they cannot have been seen to show increased levels of corticosterone and behaviours associated with stress, such as feather plucking (Vestergaard *et al.*, 1993; Vestergaard *et al.*, 1997). This is of particular relevance to our studies in pheasants as all birds were raised on grass to one year of age where dust bathing is possible and then either kept on grass or moved to concrete where the

ability to dust bathe is severely restricted. Based on the previously mentioned studies it is likely that this caused a significant stress reaction in the pheasants housed on concrete.

A further potential stressor is that of heat stress. Birds housed on concrete were subject to greater changes in ambient temperature due to the different heat absorption and heat capacities of concrete and grass. During summer this led to the concrete becoming extremely hot to the touch causing birds to shelter in the brashing and laying boxes. Heat stress has been shown to have a detrimental effect on laying characteristics such as egg number and quality in chickens (Deandrade *et al.*, 1977; Al-Saffar and Rose, 2002; Mashaly *et al.*, 2004; Pereira *et al.*, 2008). Furthermore, long term stress has negative effects on reproduction (Collu *et al.*, 1984; Smoak *et al.*, 1988; Rivest and Rivier, 1995), and so it is likely the combined stresses of poor enrichment, inability to perform natural behaviours and heat stress played a significant part in the reduction in egg number and hen condition seen in hens in these studies. However, as well as the direct effects there is also likely to be a feedback component between stress and behaviour, such that an increase in stress leads to an increase in the frequency of third party orientated stressful behaviours such as feather plucking (Allen and Perry, 1975; Blokhuis, 1986; HuberEicher and Wechsler, 1997; Aerni *et al.*, 2000; Rodenburg and Koene, 2004) and egg eating, leading to even greater stress in conspecifics and a proliferation of stressful behaviours.

These effects may operate in complex ways, for example, aside from the direct negative effects of maternal stress on egg production there are potential transgenerational effects of maternal stress, acting mostly through corticosterones, on the offspring's developing immune system (Figure 6.1). Birds housed on concrete may have been exposed to lower levels of coccidiosis and so could have had lower antibody levels at the time of laying. Any reduction in female anti-coccidia antibodies is likely to impact on their ability to transfer immunity to their offspring while at the same time a possible increase in pre-natal corticosterone could reduce the offspring's ability to mount their own immune response (Padgett, 2004; Yorty and Bonneau, 2004; Merlot *et al.*, 2008).

Once challenged these offspring are likely to be at a disadvantage as they are likely to have little or no maternally derived antibodies and, due to relatively higher corticosterone exposure, a reduced ability to produce their own immune response leading to a more severe or longer lasting infection, which is likely to manifest as a reduction in condition. However, if the maternally derived immune response is reduced, offspring will not experience such a dramatic blocking response from the mother's antibodies and so may be able to mount a more effective immune response to coccidiosis. This blocking response has been previously discussed, but in summary the maternally derived antibodies bind to pathogen antigens and prevent the offspring's immune system from reacting to them (Siegrist, 2003). On the other hand, the costs of blocking are likely to be transitory and reduce as the maternal immune response wanes, whereas there is emerging evidence that the maternally derived antibodies can potentially provide a long term beneficial "educational effect"

for the offspring leading to relatively greater antibody response once the maternal response has waned (Gasparini *et al.*, 2006; Grindstaff *et al.*, 2006; Reid *et al.*, 2006).

Future work looking at the long term costs and benefits of being raised on concrete or grass should be of great interest to scientists involved in the game and poultry industries. This study consistently found that the effects of substrate were significantly greater than that of the CoxAbic vaccine, both in terms of the direct costs to the mother of being reared on concrete, and the transgenerational effects on her offspring's health and immunity. This is especially important given the increasing drive within the game industry to sanitise the rearing process by rearing birds in wire cages during the breeding season and keeping a tight control on pathogens within the system. Based on the results presented within this thesis these actions may actually have detrimental long term effects on the offspring by removing the beneficial maternally derived antibodies and exacerbating the stress caused to the mothers by being reared in sterile conditions.

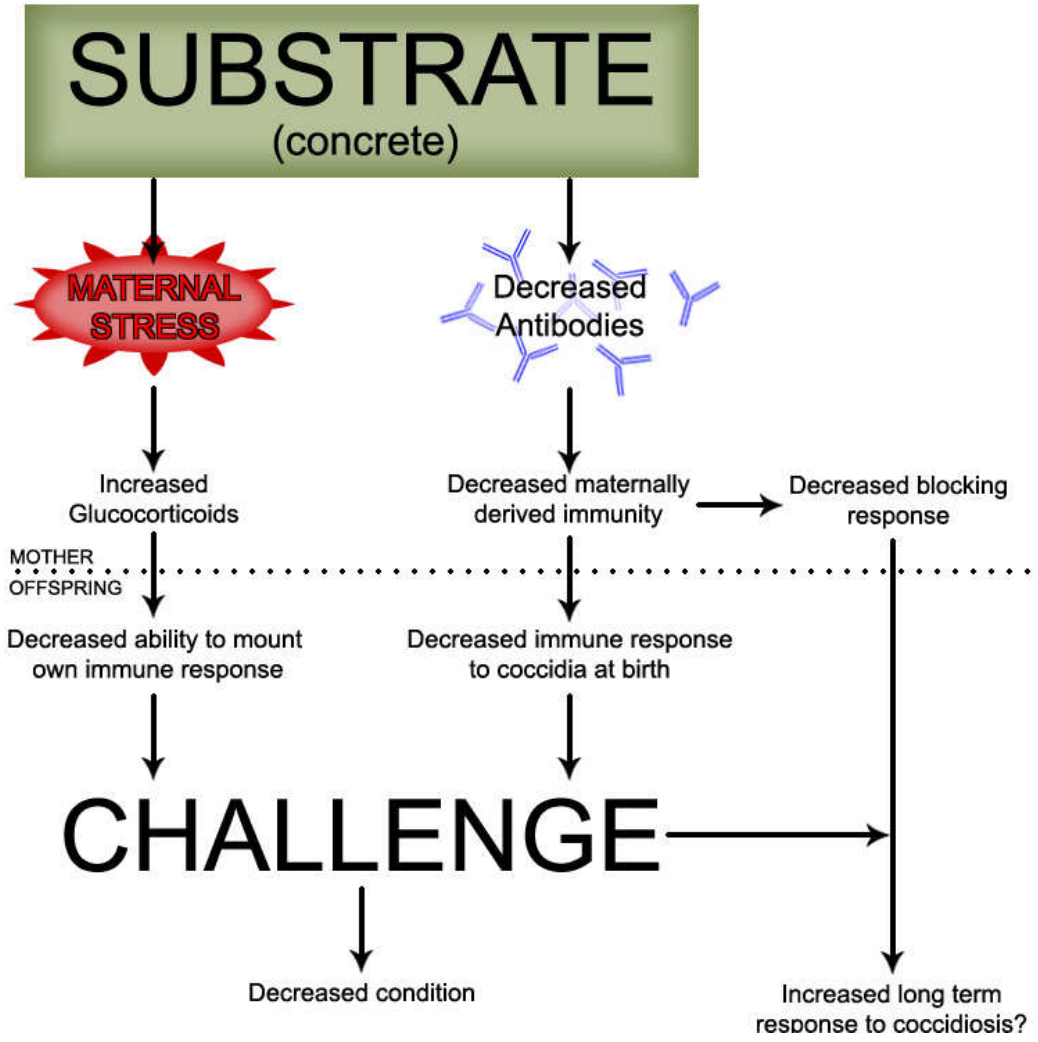

Figure 6.1: A diagrammatic representation of the effects of maternal substrate and stress on the offspring's ability to mount an immune response.

Finally, this study found that female investment in life-history traits can be further influenced by the traits of the males to which they are mated (over and above any genetic contribution they may make). These results were not consistent with the traditional view of differential allocation, in that females invested less in breeding attempts with males with larger signals that are assumed to be under sexual selection. There are a number of potential explanations, such as the difference in mating systems in these studies, the measured signals not being under sexual selection or being signals of harassment. However, further work is needed to consider how investment is affected under natural breeding situations.

In conclusion, there have been large steps forward in understanding the mechanisms of maternal effects in recent years. However, much work still remains to be done in order to understand the adaptive value of maternal effects and the relative costs and benefits both to the mother and offspring. This study attempted to answer some of these questions using the CoxAbic vaccine but instead found that rearing substrate appears to be a more potent driver of differential maternal effects in pheasants.

Chapter 7 - References

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